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Peripheral Immune Cell Abnormalities Associated with Cystic Fibrosis

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DECLARATION OF ORIGINALITY

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Emily M. Mulcahy

ABSTRACT

Cystic fibrosis (CF) is the most common life-limiting single-gene disease. It is caused by mutations to the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that is expressed on immune cells such as dendritic cells, monocytes/macrophages and lymphocytes. Lack of CFTR expression on lymphocytes and monocytes/macrophages has been reported to result in abnormal immune responses. An unresolving hyperinflammatory and T helper (Th)2/Th17 skewed immune response has also been reported in CF. We therefore hypothesised that abnormalities in both the innate and adaptive immune responses may play a pivotal role in the pathogenesis of CF lung disease.

Peripheral blood mononuclear cells (PBMC) isolated from people with CF, CF carriers and healthy age-matched controls were stained with antibodies for identification of $CD4^+$ effector (Th1, Th2, Th17) and T regulatory (Treg) ($FOXP3^+$ Treg, IL-10^+ T regulatory 1 [Tr1], transforming growth factor (TGF)β^+ Th3) cells, naïve/memory $CD4^+$ and Treg cells expressing the Th1-, Th2- and Th17-associated homing markers (CXCR3, CCR4, CCR6) and innate immune cell populations including natural killer (NK) cells, monocytes, dendritic cells (DC) and myeloid derived suppressor cells (MDSC) using multicolour flow cytometric analysis. Gene expression of the main transcription regulators of $CD4^+$ subsets and of inflammatory markers was also measured using reverse transcription–quantitative polymerase chain reaction (RT-qPCR).

Subtle changes were observed in T cell subset proportions in CF peripheral blood, with a detectable Th2 bias and a link between poor lung function and high Th17 percent. All innate immune cell proportions were altered in CF compared with healthy controls, and percentages of NK and MDSC were also correlated with lung function. CF carriers, who have only one mutated *CFTR* copy, presented with an immune phenotype similar to CF patients or intermediate between those of controls and CF patients. Because the CF carriers did not have chronic infections, this suggested that the many of the abnormal immune responses seen in people with CF may be the consequence of defective CFTR rather than of chronic infection. These findings suggest that greater emphasis should be placed on treating aberrant immune responses in CF.

CHAPTER 1

Literature Review

1.1.1 Introduction

The work described in this thesis is related to the functioning of the immune system in people with cystic fibrosis (CF). Therefore, this literature review will first discuss the disease CF, then describe the functions of the important immune cells with a focus on the subsets that we studied, before relating these functions to the situation in CF. People with CF are known to have a hyperinflammatory and dysregulated immune response involving both innate and adaptive immune cells, but detailed research in this area is limited. The innate immune cells discussed in this review include monocytes, natural killer (NK) cells, dendritic cells (DCs) and myeloid derived suppressor cells (MDSC), macrophages and neutrophils while the adaptive immune cells discussed include the $CD4^+$ effector (T helper (Th)1, Th2, Th17) and regulatory T cell (Treg) subsets ($FOXP3^+$ Treg, $IL\text{-}10^+$ T regulatory 1 [Tr1] and transforming growth factor (TGF)-β^+ Th3), $CD8^+$ T cells and B cells. Chemokine-mediated homing of immune cells and its role in disease is also reviewed. Finally the current knowledge of the role of the innate and adaptive immune responses in CF pathogenesis is discussed.

1.1.2 Cystic Fibrosis

CF is the most common life-limiting single-gene disease in Caucasians, occurring at a rate of 1 in every 2500 live births (1). In Tasmania there is a higher incidence of 1 in every 1600 live births (2), the second highest incidence in the world. CF was first recognized in the 1960s as a recessive genetic disease. At that time it presented with lung infections and deficiencies in pancreatic function and was fatal during childhood (3). The life expectancy of children with CF rose as more research was invested into understanding the pathophysiology of the disease and the full extent of the associated complications was revealed. CF is now understood to be a complex multisystem disease with additional complications often including diabetes, malnutrition, osteoporosis, arthritis, heart, kidney and liver disease and male infertility (3). The current average life expectancy of those with CF is 37 years, but it has been predicted that progressively advancing and aggressive therapeutic strategies will mean that those born in the 21st century will live to be in their 50s (4), and that there will soon be more adults than children with CF (1).

1.1.3 Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene

CF results from mutations to the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (5). The product of this gene acts as a cyclic AMP-regulated chloride channel but is also

involved in the regulation of sodium, calcium and potassium ion channels (6-8). The *CFTR* gene is located on chromosome 7 (9) and is classified as a member of the ATP-binding cassette (ABC) superfamily (10). Currently, over 1600 different mutations in *CFTR* have been identified (1), the most common of which is the deletion of phenylalanine at codon 508 known as phe508del or DF508. This mutation occurs in around 70% of people with CF worldwide, although the frequency varies in specific ethnic groups and geographic regions (11).

The different mutations of the *CFTR* gene result in diverse phenotypes (11) depending on whether the mutation affects synthesis, processing or transport of the protein or functionality of the resultant channel. *CFTR* mutations are classified into six groups (12), which include defective protein synthesis (class 1), abnormal or defective protein processing or transport (class 2), defective channel regulation (class 3), decreased channel conductance (class 4), reduced level of synthesis, maturation or transport of the protein (class 5) and decreased protein stability (class 6). The DF508 mutation of *CFTR* produces a protein that often retains a considerable amount of function as a chloride channel, but which is misfolded, resulting in ubiquitination that marks it for degradation before it can traffic to the epithelial cell surface (class 2) (13). Some mutant forms of the CFTR protein, such as A455E, reach the cell surface and retain moderate functionality as chloride channels (class 4), which may be a contributing factor to the occurrence of clinically milder forms of CF (14). Further investigation into the phenotype–genotype relationship has revealed that there is a strong correlation between genotype and pancreatic disease but minimal correlation with lung disease (11, 15, 16). This observation may suggest that environmental and additional genetic factors play a greater role in the development of lung disease (17).

1.1.4 Effects of *CFTR* Gene Mutations

The main cell type to express CFTR are epithelial cells, of which the most commonly affected in people with CF are those in the sweat glands, salivary glands, airways, nasal epithelium, bile ducts, pancreas, vas deferens (in males) and intestinal epithelium (18). The organ most affected by defective CFTR is the lungs, which are frequently chronically infected, leading to the main cause of morbidity and mortality (90%) in people with CF, respiratory failure (19). However, more recently it has also been shown that CFTR is expressed in other cell types including those of the immune system such as antigen presenting cells (APC) and T lymphocytes, although it is not yet known whether all or only some specific T cell subsets

express CFTR. Evidence suggests that expression of mutant CFTR on these immune cells could result in immunoregulatory defects (20-23).

The means by which CFTR mutations cause lung disease have not yet been fully elucidated, but it has been postulated to result from a combination of different mechanisms. These mechanisms include impaired regulation of the fluid volume or composition on the surface of the airways (18, 24), increased binding of or decreased ability to clear pathogens such as *Pseudomonas aeruginosa* (25) and increased levels of proinflammatory cytokines in the airways (26, 27). The normal function of mucus in healthy lungs is to trap inhaled particles in order to keep the airways clean. Healthy mucus has low viscosity to allow ciliary beating, which promotes movement of the mucus and attached particles towards the mouth to be either swallowed or expelled. In people with CF, impaired fluid levels/composition in the airway result from a decreased level of chloride secretion and increased absorption of sodium by airway epithelial cells. This results in a lower water content in the mucus, causing its relative dehydration in the airways and mucus retention, resulting in a reduced ability to clear inhaled particles (28). This impaired ability to clear mucus and pathogens such as *P. aeruginosa* results in infection and inflammation then bronchiectasis, leading to progressive loss of lung function and eventually death. A vicious cycle of infection and inflammation occurs whereby harmful products such as proteases, elastases and oxidants, mainly produced by neutrophils, are released, causing inflammation that predisposes to further infection (29).

It is not entirely clear whether it is inflammation or infection that occurs first in the lungs of CF patients. Muhlebach *et al.* (30) investigated bronchoalveolar lavage fluid (BALF) from paediatric CF patients with and without lower respiratory tract infection as well as healthy paediatric controls and revealed an increased level of neutrophils in both infected and non-infected CF patients compared with healthy controls, indicating a proinflammatory state regardless of infection. Khan *et al.* (31) has also shown increased levels of neutrophils in BALF from CF infants without any detectable bacterial, viral or fungal infection. However, these studies did not exclude patients who had experienced infection prior to the study. In contrast, a study by Armstrong and colleagues comparing BALF from CF infants who were yet to experience any detectable infection with that of healthy controls showed normal levels of neutrophils in the CF patients. However, this study did not exclude the 30% of controls who tested positive for or developed symptoms of respiratory tract infection within 48 hours following bronchoscopy. A study by Hubeau *et al.* (32) that investigated lung samples from CF and non-CF foetuses that had therefore not experienced any infection showed that in CF

there was an increased level of macrophages, a source of neutrophil chemoattractants, indicating the existence of an inherently proinflammatory state even before exposure to pathogens. In summary, much of the evidence strongly supports the theory of a hyperinflammatory state in CF prior to any infection.

1.1.5 Lung Infection in Cystic Fibrosis

A significant clinical feature of CF is the development of chronic lung infection. *Staphylococcus aureus* and *Haemophilus influenzae* have traditionally been considered the most common initial infecting pathogens dominating in the lungs of CF children, with a dominance of *P. aeruginosa* and *Burkholderia cepacia* developing with increasing age (33, 34), as shown in Fig 1. However, with the advent of molecular identification techniques it has become apparent that CF lungs are colonised with a much more complex population of pathogens. While this was originally shown by Rogers and colleagues using 16s ribosomal DNA length heterogeneity PCR and 16s ribosomal DNA terminal restriction fragment length polymorphism (35), this has since been supported with other studies revealing an intricate community consisting of bacterial, viral and fungal infections (36-38). However, *P. aeruginosa* is still widely considered the most frequent, chronic and major pathogen, occurring in an estimated 80% of adults with CF (39).

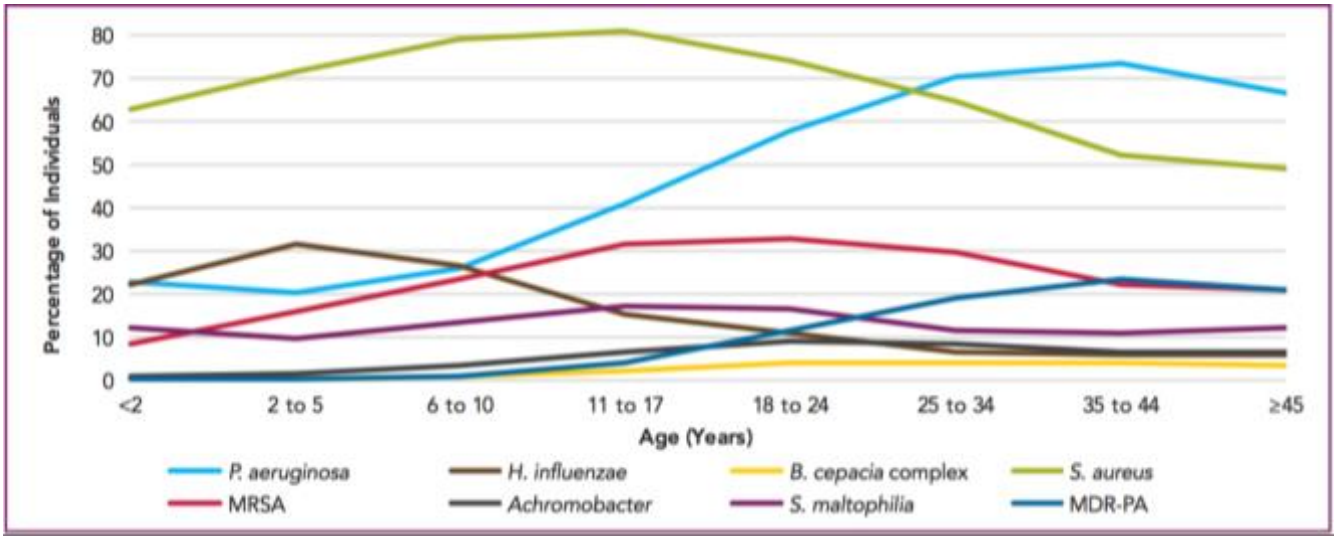


Fig. 1. Development of the microbial community within the CF lung. Figure illustrates the incidence of the primary respiratory pathogens in CF in relation to age. Figure taken from the Cystic Fibrosis Foundation's annual data report (40).

While bacterial infection is a primary focus in regards to treatment of CF, viral infection also plays a significant role in deterioration of lung function. Respiratory syncytial virus (RSV) and influenza have been shown to have the most deleterious effects on the lungs (41), although other viruses such as parainfluenza, rhinovirus and adenovirus are also commonly detected in CF (42). Viral infections in CF have been associated with poorer lung function and increased frequency and duration of hospital admissions (43, 44). Of particular interest is the theory that viral infection, particularly RSV, facilitates bacterial infections such as with *P. aeruginosa*. In a 25 year retrospective study in Denmark, Johansen and Hoiby (45) found that the first detection of *P. aeruginosa* most commonly occurred during peak RSV season. However, this study needs to be considered cautiously given that the peak season for influenza is very similar to that of RSV. A study of six CF patients by Collinson and colleagues reported that five developed their first *P. aeruginosa* infection while experiencing symptoms of an upper respiratory tract infection. Armstrong *et al.* (41) also demonstrated that a viral infection was detected in BALF during 50% of exacerbations. The mechanism behind the link between viral and bacterial infection is yet to be elucidated, although it has been suggested that viral infection disrupts the epithelial cell membrane, thus facilitating greater bacterial adherence (43). This is supported by a study undertaken by Fainstern *et al.* (46) in which *S. aureus*, *H. influenzae* and *S. pneumoniae* all showed increased adherence to pharyngeal cells after participants received a live attenuated influenza vaccination.

With the improvement of culture and molecular techniques it has become apparent that fungi are also dominant pathogens within the CF lung. Nagano *et al.* (47) have shown that although with traditional culture methods fungi could be detected in only 18% of CF sputum samples, the use of more specific culture techniques resulted in the detection of fungi in 78% of the samples. The use of next-generation sequencing analysis of the CF lung microbiome further supports this and has made it evident that fungi are indeed abundant within the CF lung (37, 48). This could suggest that the impact of fungi within the CF lung has previously been underestimated. *Aspergillus fumigatus* is the most commonly isolated fungus, present in an estimated 50% of CF patients (49). Colonisation by this fungus can lead to allergic bronchopulmonary aspergillosis (ABPA), an allergic inflammatory condition which has been shown to be associated with worse lung function and a more rapid decline (50). ABPA has been associated with a predominantly Th2-type immune response in CF (51, 52) which has previously been shown to have negative impacts on the clearance of pathogens such as *P. aeruginosa* (53). Reports are also beginning to emerge that chronic *A. fumigatus* infection, even without ABPA, can result in worsened lung function (54, 55). In addition to this it has

also been shown that colonisation with *A. fumigatus* increases the risk of *P. aeruginosa* infection (56). Given the serious impact of *P. aeruginosa* in CF, this evidence suggests that fungal infections should be treated more seriously in management of CF. However, further research is required to determine the full extent of fungal infections and their interaction with bacterial infections in CF.

1.1.5.1 *Pseudomonas aeruginosa* Infection

P. aeruginosa is a Gram-negative aerobic bacillus. It is ubiquitous throughout the environment, mainly in environments such as soil and water, and is an opportunistic pathogen primarily affecting immune-compromised individuals such as those with burns, foreign bodies and those undergoing chemotherapy or invasive surgery, as well as those with CF. It is known to be the dominant pathogen in the CF airways and the most significant influence on progression, prognosis and ultimately mortality of those with the disease (57, 58). Eighty percent of adults with CF become chronically infected with *P. aeruginosa* (59), the most common source of which is the environment .

P. aeruginosa has a high level of genetic diversity (60), which contributes to its ability to remain in the lungs of CF patients and to maintain its pathogenicity. It is able to metabolize many carbon and nitrogen sources to provide energy (60), and although it is aerobic it is able to adapt to an environment with limited or no oxygen (61). This characteristic facilitates lung colonization in those with CF because they have thick mucus in their airways that limits the diffusion of oxygen. *P. aeruginosa* also possesses many virulence factors that mediate infection and lung tissue damage, such as pili, flagella, lipopolysaccharide (LPS), elastases, proteases, phospholipase C, hydrogen cyanide, exotoxin A, exoenzyme S and pyocyanin (60, 62). Once established in the lungs, *P. aeruginosa* undergoes many phenotypic changes associated with the selective pressures experienced within the CF lung, leading in many cases to the development of mucoid strains that are highly resistant to multiple antibiotics (63).

1.2.1 Innate and Adaptive Immunity

The innate immune response is the body's first line of defence against infection, and acts immediately, recognising invasion by foreign microbes nonspecifically through detection of common microbial components. The adaptive immune response, which is delayed relative to the innate response, recognises foreign invaders through antigen-specific receptors on immune cell surfaces. After stimulation of these receptors, adaptive cell-mediated and

humoral immune responses are mounted, facilitated by T lymphocytes ($CD8^+$ and $CD4^+$ T cells) and B lymphocytes, respectively.

1.2.2 Innate Immune Cells

1.2.2.1 Monocytes

Monocytes are a population of leukocytes that develop in bone marrow, circulate through blood and are the precursors of macrophages, DCs and osteoclasts. Monocytes have been implicated in many disease pathologies and, because recent research has indicated the presence of several distinct macrophage phenotypes, understanding the role of each individual subset in disease states has become even more important. Altered monocyte subsets have been implicated in an array of diseases including rheumatoid arthritis (64), systemic lupus erythematous (65), bacterial infections (66, 67), human immunodeficiency virus infection (68), asthma (69) and Crohn's disease (70). Monocytes were traditionally categorised into two subsets, based on CD14 and CD16 expression, known as 'classical' and 'non-classical' monocytes, first identified by Passlick *et al.* (71). While distinct functions and phenotypes were identified for these two subsets (72, 73) it became apparent that there was greater heterogeneity amongst the non-classical monocytes. It is now widely accepted that monocytes consist of three distinct subsets that are still identified based on expression of CD14 and CD16. However, more defined flow cytometry gating parameters have allowed the isolation of an intermediate population (74). Because of inconsistent flow cytometry gating methods and isolation techniques used in different studies there are still some discrepancies in the reported functions of these monocyte subsets.

The primary population, termed 'classical' monocytes and comprising approximately 80–90% of the monocyte population, are $CD14^{hi}CD16^{lo/neg}$. Classical monocytes are involved in injury and inflammation and are recruited to inflamed tissue. The homing of these cells to affected tissues occurs because of increased levels of the chemokine CCL2 at sites of inflammation and the high level of expression of the chemokine receptor CCR2 on classical monocytes. These cells are primarily involved in phagocytosis of pathogens and damaged cells, and are considered to be proinflammatory because they produce interleukin (IL)-6, IL-8, and the chemokines CCL2 and CCL3 (75). This population has not been shown to infiltrate tissues under homeostatic conditions but is able to infiltrate tissues and differentiate into DCs and macrophages under inflammatory conditions (76).

'Intermediate' monocytes are $CD14^{int}CD16^{int}$ and comprise approximately 5% of the monocyte population. It is debated whether intermediate monocytes are a distinct population or a transitional population between classical and non-classical monocytes. Using hierarchical clustering, Cros *et al.* (77) demonstrated that classical and intermediate monocytes were closely related, while non-classical monocytes clustered separately and were more distantly related. However, there is stronger evidence to support a closer link between intermediate and non-classical monocytes (78, 79), and this relationship is present in rhesus monkeys, which possess homologous monocyte subsets (80). Intermediate monocytes are involved in antigen presentation and parasite recognition, as indicated by their high levels of expression of major histocompatibility complex (MHC) II-related processing and presentation genes (78, 79). It is recognised that this subset produces a moderate level of proinflammatory cytokines such as tumour necrosis factor (TNF), IL-1β and IL-6 (66, 81). While they express low levels of the chemokine receptor CCR2, they also express CCR5, which facilitates homing of these cells in response to the chemokines CCL3, CCL4 and CCL5 that are highly expressed in inflamed tissues (82).

'Non-classical' monocytes are $CD14^{lo}CD16^{hi}$ and comprise approximately 10–15% of the monocyte population. They express low levels of the chemokine receptors CCR2 and CCR5 but high levels of CX3CR1, which facilitates a prolonged patrolling behaviour along the endothelium that involves this population in local innate immune surveillance (78). Because of their lower expression of CD14, a coreceptor for detection of bacterial LPS, non-classical monocytes are less responsive to bacteria than the classical and intermediate subsets but are more responsive to viral pathogens (77). This population is primarily responsible for infiltrating tissues, including the lungs, under homeostatic conditions, and differentiating into tissue-resident macrophages (83). However, they have also been shown to be able to traffic to inflamed tissue (76). There is still disagreement about the cytokine profile of non-classical monocytes, with studies indicating this population is a poor producer of proinflammatory cytokines such as TNF (77). However, these studies have used isolated cells that have been manipulated extensively, while Belge *et al.* (84), who used a minimally invasive whole blood intracellular staining technique, showed that non-classical monocytes produce a high level of TNF. This finding was further supported by the results of Wong *et al.* (79).

The effects of *CFTR* mutations in CF monocytes have been well documented (85-87) with reports describing defects in adhesion (85), IL-8, TNF, proteinase and elastase secretion (88-91), and complement-mediated phagocytosis of pathogens such as *P. aeruginosa* (92). No

studies have yet investigated the three monocyte subpopulations and their role in CF pathogenesis. Considering the distinct functions of each subset, investigating this could provide a much more detailed understanding of monocyte dysfunction in CF.

1.2.2.2 Neutrophils and Macrophages

CF is characterised as a neutrophilic airway inflammatory condition (93, 94) in which there is an exaggerated influx of neutrophils to the lungs. As discussed in section 1.1.4, it has not been fully elucidated whether increased neutrophil levels are inherent to CF or are a result of infection. While neutrophils can be considered beneficial for combatting pathogens, in CF they can also become damaging to lung tissue through release of toxic granule contents accumulating over long periods of time (95). Studies have shown that CFTR affects microbicidal activity in neutrophils by reducing chloride anion concentration, which in turn reduces the ability of the phagosome to produce hypochlorous acid, a critical component in bacterial killing (96-98). Neutrophils have also been shown to have reduced phagocytic capacity in young CF patients (99). However, in contrast to this, another study by Kingma *et al.* (100) has shown no change in neutrophil phagocytic capacity in CF newborns, suggesting that this manifestation may be influenced by the development of infection. There are a significant number of studies, as reviewed by Hartl *et al.* (95), which have all demonstrated the link between neutrophils and/or detection of their products (proteases, oxidants and proteins) and reduced lung function. This evidence supports a significant role for neutrophils in CF lung disease and indicates the need for greater understanding of the molecular mechanisms driving their accumulation and modulating their function.

While there are various populations of tissue macrophages, alveolar macrophages are of particular interest in CF as they are a key cell type involved in the initial defence against pathogens in the lung. Alveolar macrophages are phagocytes residing within the lungs that when activated in response to pathogens eliminate bacteria, dead cells and debris, and promote the influx of neutrophils and the activation of DCs and T cells, thereby connecting the innate and adaptive immune responses. However, alveolar macrophages are poorer presenters of antigen to T cells and have reduced expression of costimulatory molecules as an adaptation to reduce lung inflammation to innocuous microbes (101). Alveolar macrophages have been shown to be increased in infection-free CF children (102), suggesting that this elevation may be inherent to CF rather than infection mediated. This has been further supported by a study involving exposure of *cftr* knockout mice to *P. aeruginosa,* in which alveolar macrophages were shown to directly contribute to the exaggerated immune response

seen in CF. Alveolar macrophages, like that of monocytes, can be divided into two subsets known as 'classically activated' or 'M1' macrophages that produce proinflammatory cytokines and are polarised by bacterial infection and IFNγ, and the 'alternatively activated' or 'M2' macrophages that promote tissue remodelling and repair after inflammation. M2 macrophages are polarised by Th2-associated cytokines such as IL-4 and IL-13 (95). An appropriate immune response requires the balanced coordination of both of these subsets. In CF, the polarisation of macrophages has been investigated but the results are inconclusive. Murphy *et al.* (103) have shown a polarisation towards an M2 phenotype in BALF of *P. aeruginosa* infected CF patients, while Tarique *et al.* (104) have shown an M1 phenotypic bias in CF peripheral blood. Meyer *et al.* (105) also shown using a CF mouse model that both the M1 and M2 phenotypes were upregulated in response to LPS from *P. aeruginosa*. These conflicting findings suggest that further research is required to understand the role and extent of macrophage polarisation in CF.

Macrophages that circulate through blood and lymph are also important because they act not only as part of the innate immune system, through direct clearance of pathogens, but also as part of the adaptive immune system, through presentation of antigens to Th cells. Evidence now suggests that defective CFTR may inhibit macrophage activation and affect macrophage pathogen killing. This is supported by a study showing reduced numbers of activated macrophages in CF (as defined by F4/80 and MHC-II exppression) (106), although it is known that macrophage numbers are increased in CF lungs (107). It has been suggested that defective CFTR causes an accumulation of intracellular Ca^{2+} that is sufficient to alter gene expression (108) and hence alter processes involved in macrophage activation. This has been supported by a study showing that wild-type macrophages treated with CFTR inhibitors displayed increased intracellular Ca^{2+} levels (109). However, further investigation into alterations in macrophage gene expression in relation to function is required. Nevertheless, this suggests another immune cell that may be contributing to an ineffective immune response and clearance of pathogens in people with CF.

1.2.2.3 Dendritic Cells

DCs are professional antigen presenting cells that have the primary function of presenting antigen to T cells and aiding in the differentiation of B cells, thus creating a connection between the innate and adaptive immune systems. DCs develop in bone marrow and travel in blood throughout the body. Once they capture antigen for presentation they mature and travel

to the lymph nodes where they can encounter T cells for presentation (110). Mature tissue-resident DCs can also be found in tissues, particularly at mucosal sites (111).

There are two types of DCs present in human peripheral blood under normal conditions, known as 'conventional/classical' DCs and 'plasmacytoid' DCs (112). Conventional DCs can be further subdivided into two subsets based on CD1c and CD141 expression. However, in the presence of GM-CSF and IL-4 during inflammation, a third type of DC develops from $CD14^+$ classical monocytes, termed 'monocyte-derived' DCs, a proinflammatory subset secreting TNF (113).

Much of the research investigating DCs in CF has been undertaken using mouse models. These studies have shown a downregulation of expression of costimulatory molecules such as CD40, CD80 and CD86 (114), reduced maturation capacity and reduced expression of genes associated with membrane structure and lipid metabolism (23, 114-118), particularly in response to specific pathogens such as *P. aeruginosa* (23, 117) and *B. cepacia* complex (118). This suggests that dysfunction of DCs in CF may be influenced by a combination of both intrinsic and infection-mediated effects and, given that antigen presentation molecules are affected, this could have negative impacts on the generation of appropriate adaptive immune responses.

1.2.2.4 Myeloid Derived Suppressor Cells

MDSC are a heterogeneous population of immature innate myeloid cells with a potent suppressive capacity, which are thought to be precursors to monocytes, dendritic cells and granulocytes at various stages of differentiation (119). Because this population has been identified only recently and is heterogeneous, identification of distinct MDSC phenotypes is ongoing. However, two commonly accepted subsets have thus far been identified: monocytic MDSCs ($lineage^{neg}HLA\text{-}DR^{neg}CD33^+CD11b^+CD14^+$) and granulocytic MDSCs ($lineage^{neg}HLA\text{-}DR^{neg}CD33^+CD11b^+CD14^{neg}CD14^{neg}CD15^+CD66b^+$) (120). MDSCs are believed to exert their suppressive capacity via cell contact dependent mechanisms such as interaction with cell surface receptors and the release of short lived soluble mediators (119).

While expansion of MDSCs was originally described in cancer (121), it has become apparent that MDSCs play a role in many disease states including tumour formation (122), inflammatory diseases (123), and viral and parasitic infections (124, 125). Just one study, by Rieber and colleagues (126) has investigated MDSCs in CF and this focussed only on the granulocytic MDSC population in peripheral blood. Granulocytic MDSCs were shown to be increased in CF, particularly in response to *P. aeruginosa* infection, and their level positively correlated with lung function. This is supported by a study involving LPS-induced MDSCs in

non-CF lung bacterial infection that showed that these cells are able to both suppress T cell responses and efferocytose apoptotic neutrophils (127). This may indicate that MDSC are beneficial in CF, because a reduction in the damaging hyperinflammatory T cell and neutrophil responses may improve lung function.

1.2.2.5 Natural Killer Cells

NK cells were first identified by Keissling and colleagues in mice in 1975 (128). NK cells are now known to be a cytotoxic innate immune cell population that are primarily able to kill virus infected, stressed or non-self cells. In addition, they are able to influence the immune response through the production of cytokines such as interferon (IFN)γ, TNF and granulocyte–macrophage colony stimulating factor (GMCSF) and chemokines such as CCL3 and CCL4. Much of the literature describes two primary peripheral blood NK subsets based on CD16 and CD56 expression. $CD56^{bright}$ NKs are classified functionally as the cytokine-producing population while $CD56^{dim}$ NKs are classified as the cytotoxic population (129), although recent *in vitro* studies have shown that under certain conditions each of these populations can be stimulated to perform the alternate function of either cytokine production or cytotoxicity (130, 131). More recently a third NK cell subset has been identified that is low in expression of both CD56 and CD16 and was shown to have moderate cytotoxic capacity and a moderate ability to produce cytokines such as IFNγ (132, 133). While $CD56^{dim}$ NKs dominate in the periphery, the $CD56^{bright}$ population tends to dominate in tissues, especially during inflammation (134). While there have been suggestions that NK cells can be further differentiated into five subsets based on further functional testing, this has yet to be confirmed (135).

NK cells are essential in the lungs because this is a major site of entry for pathogens, where rapid responses are required to limit their spread. However, aberrant or reduced functioning of NK cells can contribute to immune pathologies in conditions such as autoimmunity, tuberculosis, influenza and asthma (136, 137).

NK cells in CF have gained very little attention, with only a small number of studies investigating their frequency in peripheral blood (138-140). While all of these studies indicated a decrease of NK cells in CF peripheral blood, no further investigations of NK subpopulations or function were undertaken. NK cells have been implicated in not only viral infections of the lungs but also bacterial infections, where they are believed to play an early role in controlling bacterial dissemination (141).

1.2.3 Adaptive Immune Cells

1.2.3.1 B Cells

B cells form the humoral branch of the adaptive immune response and act through the production of antibodies/immunoglobulin, usually regulated by Th cells, which in turn can neutralise pathogens or tag them for destruction by other immune cells. There are three primary B cell subsets known as follicular (FO) B cells, marginal zone (MZ) B cells and B1 B cells. FO B cells are the most abundant and circulate through lymphoid follicles of lymph nodes and spleen and are specialised for antibody production in a T-cell dependent manner. MZ B cells are located in the marginal zones of the spleen where they are ideally situated to respond to blood borne antigens while B1 B cells are located at mucosal sites where they are positioned to combat invading environmental pathogens (142). However, B1 B cells are a less well characterised population in humans (143).

There has been very little investigation of B cells in CF, with the few available studies indicating a dysregulation in the differentiation of B cells (144) as well as secretion of antibody (145), specifically in response to *A. fumigatus* antigens (146). While levels of B cells have not been specifically investigated in the CF lung, a study by Niell *et al.* (147) has shown an increase in BAFF, a B cell growth factor that promotes the maturation and antibody production of B cells, and an increase in the B cell chemoattractants CXCL13, CCL19 and CCL21 in *P. aeruginosa*-negative CF paediatric lungs. Consequently, this may suggest that B cells and their associated antibodies may be increased in CF lungs, which is supported by a study also indicating a dominance of B cells in CF nasal polyps (148). Dysregulation of immunoglobulins (Ig) secretion by B cells has also been shown in CF, with reports of increased IgG, IgM and IgA in the serum of adult CF patients compared with healthy controls (149) and increased IgM and IgA in paediatric CF patients compared with healthy controls (150). Of importance, IgG has been shown to be associated with poorer lung function and worsened disease severity in both adults and children with CF (149, 150) while IgM has been shown to be associated with worsened disease outcome in paediatrid CF patients (150). While the levels of IgE are not altered in the general CF population, elevated IgE has been recorded during the development of the allergic response to *A. fumigatus* known as ABPA, and has been associated with decreased lung function (151, 152). While the mechanisms behind immunoglobulin dysregulation are poorly understood, they are likely to be related to dysregulation of Th cell populations, while the evidence suggests that B cells and their associated antibodies may play a role in CF pathogenesis, particularly in people with ABPA.

1.2.3.2 $CD8^+$ T Cells

$CD8^+$ T cells or cytotoxic T cells are T lymphocytes that are able to directly kill cells, particularly those infected with intracellular pathogens, which express foreign antigen in the context of MHC class I molecules. $CD8^+$ T cells can eliminate these cells either through the release of cytotoxic granules, release of cytokines or via Fas/FasL interactions that result in apoptosis of the target cell (153). There has been limited investigation of $CD8^+$ T cells in CF with Hauser *et al.* (140) demonstrating a decrease of these cells in CF peripheral blood and Xu *et al.* (115) demonstrating a dysregulation of cytokine secretion in these cells in response to RSV infection in a CF mouse model. Given that it is known that CFTR is normally expressed in $CD3^+$ T cells (22) and that dysfunctional CFTR affects $CD4^+$ T cells and antigen presenting cells, which heavily influence $CD8^+$ T cell responses, a possible role for CD8 T cells in CF pathogenesis may exist.

1.2.3.3 $CD4^+$ T Cells

The adaptive immune system is a highly specialised and specific system defending the host against invading pathogens. $CD4^+$ T cells are a specialised cell type that coordinate and regulate various aspects of the immune response via the production of specific cytokines, and have varying effects including downregulation of the immune response and activation of cytotoxic T cells, B cells, innate immune cells and nonimmune cells (154). $CD4^+$ T cells have been categorised into two distinct subsets known as T helper (Th) 1 and Th2 based on their cytokine profiles (cytokines required for their differentiation and the cytokines they produce), functional capabilities, and their transcriptional regulators (155). They exhibit an inverse relationship, with one or the other dominating in response to a particular pathogen. $CD4^+$ T cells orchestrate the type of immune response elicited, depending on the type of invading pathogen. More recently, additional $CD4^+$ T cell subsets have been described, including Th17 and regulatory T cells (natural Treg [nTreg], induced Treg [iTreg], T regulatory 1 [Tr1] and Th3) (Fig. 2).

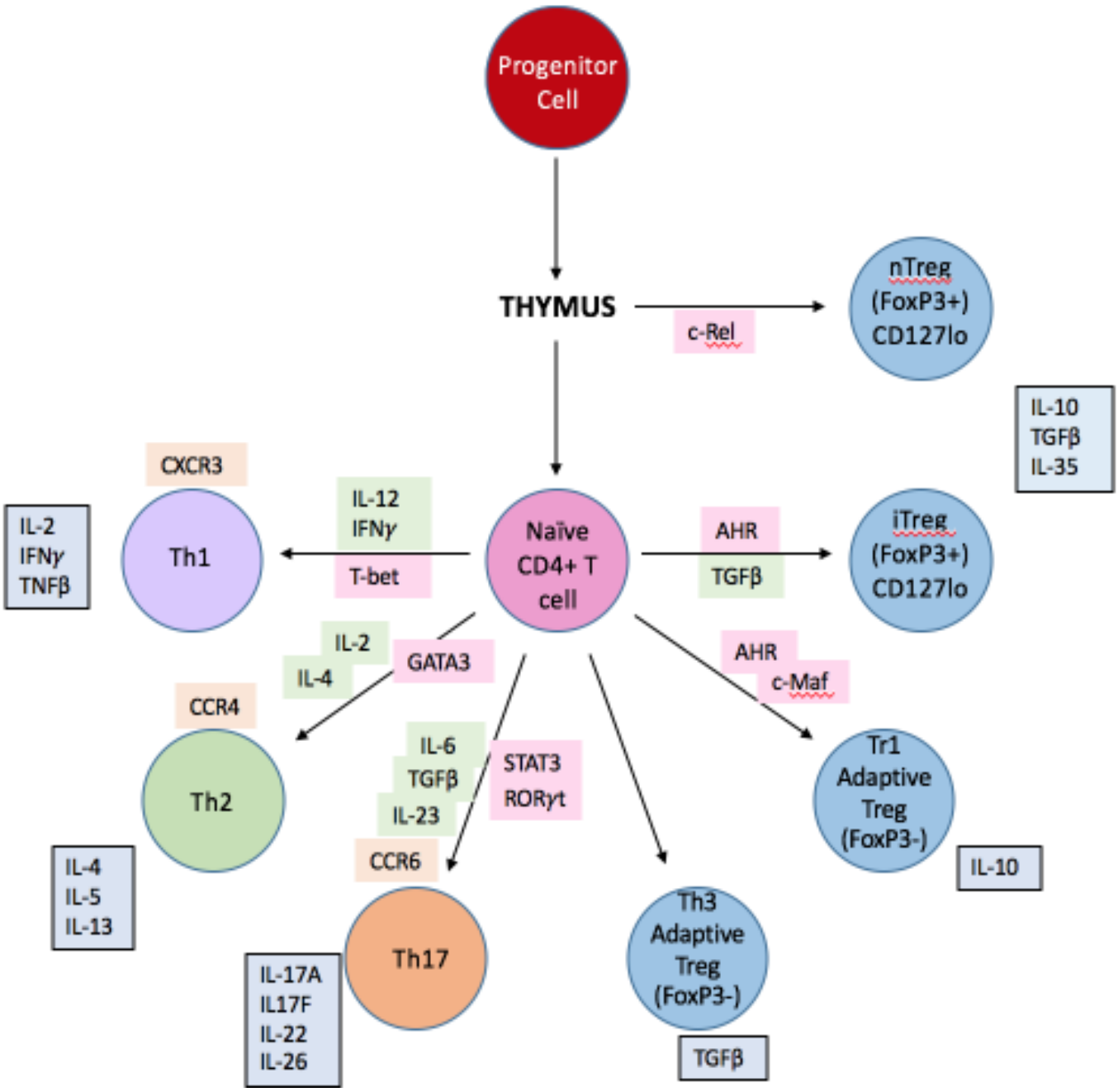


Fig. 2. Differentiation pathways of CD4⁺ T cell subsets. Major transcriptional regulators (pink boxes), main cytokines required for differentiation (green boxes), signature cytokines produced (blue boxes) and associated chemokine receptors (orange boxes) are shown. Original figure.

Naïve T helper cells are functionally immature precursor cells that have migrated from the thymus to the peripheral lymphoid organs and are capable of producing only IL-2 (156). Antigenic stimulation is required for these naïve cells to differentiate into different $CD4^+$ effector cells with distinct functions. Antigenic stimulation of a naïve T cell involves binding of the T cell receptor (TCR)-CD3 complex to the antigen–MHC II presented on APCs, with the additional interaction of costimulatory molecules. This in turn leads to the activation of downstream signalling pathways resulting in the proliferation of specific $CD4^+$ subsets (157). Differentiation of $CD4^+$ subsets is influenced by many factors including the structure, dose and route of entry of antigen (158, 159), the strength of TCR signalling (160), the presence of costimulatory molecules (158, 161), the type of APC (162, 163), the stimulation of pattern-recognition receptors by pathogen-associated molecular patterns, and genetic factors. However, the most influential factor is the cytokine environment (164). Initially, cytokines in the microenvironment are produced by APCs and other cells of the innate immune system, but once T cells begin to differentiate they can initiate the production of cytokines, which results in a positive feedback loop to enhance their own survival and production.

1.2.3.4 T Helper 1 and T Helper 2 Cells

Th1 cells are involved in cell-mediated immunity and produce IL-2, IFNγ and lymphotoxin β. These signature cytokines mediate the activation of macrophages and delayed-type hypersensitivity responses (156). This type of immune response is involved in defences against intracellular pathogens including viruses, bacteria and protozoa (158), and promotes antibody-isotype switching to IgG2a in mice (156) and IgG3 in humans (165). The cytokines IL-12 and IFNγ are required for the differentiation of Th1 cells (166, 167), and their master transcriptional regulator is the T-box transcription factor (T-BET). T-BET is known to be highly dependent on signal transducer and activator of transcription (STAT)1, which is activated by IFNγ (168) and must function in collaboration with STAT4, which requires IL-12 for activation, for full differentiation of Th1 cells (169). The inverse relationship seen between Th1 and Th2 immune responses is mediated largely by T-BET through multiple mechanisms. Predominantly, T-BET upregulates the production of IFNγ which inhibits the development of the Th2 subset (170). It also inhibits Th2 development through suppression of IL-4 expression, which consequently impairs the functioning of GATA-binding protein 3 (GATA3), the major transcriptional regulator of the Th2 subset (171).

Th2 cells are involved in the humoral immunity that occurs in response to extracellular pathogens such as helminths, and are important in allergic responses (155). They produce IL-

4, IL-5 and IL-13, which promote proliferation and differentiation of B cells and antibody-isotype switching to IgG1 and IgE (156) in mice and IgG4 and IgE in humans (165). IL-2 and IL-4 are the major cytokines required for Th2 differentiation (172, 173) and GATA3 is their main transcriptional regulator. GATA3 is not able to regulate the differentiation of Th2 cells by itself but requires STAT6 (174), which is activated by IL-4 and which then upregulates expression of GATA3 (175). Both STAT3 and STAT5 have also been shown to be important in the differentiation of Th2 cells, with STAT3 assisting STAT6 (176) and STAT5 working in close collaboration with GATA3 (174).

1.2.3.5 T Regulatory Cells

Although the classification of Treg cells is still controversial, they can be divided into three separate types known as natural, induced and adaptive Tregs. Natural Treg cells (nTreg) are released from the thymus as a fully differentiated cell type expressing the transcription factor forkhead box P3 (FOXP3), while iTreg cells are induced in the periphery after specific antigen exposure to differentiate into FOXP3-expressing regulatory cells (157). However, the majority of $FOXP3^+$ Treg cells are nTreg cells that develop soon after birth (177). The FOXP3 transcription factor regulates the suppressive capacity of Treg and iTreg cells and is essential for appropriate functioning and development of these cells (178). It has also been shown to be essential for maintaining immune homeostasis (179), with a deficiency in FOXP3 resulting in a fatal autoimmune-like disease because of overactive T cells, and FOXP3 overproduction resulting in reduced levels of T effector cells with impaired function (180). The expression of FOXP3 in non-regulatory cells has also been shown to result in the gain of suppressor function by peripheral activated T cells (178), indicating its importance in the function of these cells. Treg cells are vital in the regulation of the immune system and the induction of tolerance. They regulate many cell types including other T cells, B cells, NK cells and DCs. Regulation of T cells, both $CD4^+$ and $CD8^+$, occurs through inhibition of proliferation through contact dependent mechanisms and production of immunosuppressive soluble factors such as cytokines, although the specific mechanism are still under debate (181). nTregs regulate B cells by preventing immunoglobulin production, NK cells by preventing their cytotoxic activity, and DCs by preventing their maturation (182, 183). The major effector cytokines of nTreg cells include IL-10, transforming growth factor (TGF)β and IL-35 (184-186), which are able to turn potentially self-reactive T cells into regulatory cells that in turn can then inhibit any remaining self-reactive cells (187).

The initial differentiation of nTreg cells begins from those cells that have a TCR with high affinity for self antigen. This differs from the process for other $CD4^+$ subsets, which are eliminated by negative selection if they express high affinity for self antigens. The transcription factor c-REL is predominantly responsible for the differentiation of nTregs (188, 189), however, it is the production of IL-2 and/or IL-15 that induces the FOXP3 expression that subsequently maintains the suppressive behaviour of the nTreg cells (190). In mouse models it has been shown that an absence of c-REL results in a reduction of the number of nTregs to just 15% of wild type (189).

Induced Tregs are CD4 cells that have differentiated into $FOXP3^+$ regulatory cells in the periphery in response to cytokines such as IL-12 and TGFβ (154). It has also been shown in mice that TGFβ is the main cytokine required for iTreg differentiation (157, 191). It activates Smad2 and Smad3, which then induce FoxP3 expression (192). Investigation of iTreg differentiation has revealed that c-REL is involved in expansion of these cells but is not critical for their differentiation (189).

Adaptive Treg cells are believed to have an origin distinct from that of $FOXP3^+$ Treg cells (193). They do not express FOXP3, but exert suppressive behaviour via IL-10- and TGFβ-dependent mechanisms (194). Two types of adaptive Treg cells have been described: IL-10-producing Tr1 cells and TGFβ-producing Th3 cells (195). While nTreg cells are mainly responsible for the maintenance of self-tolerance, Tr1 cells are responsible for the regulation of inflammation-dependent adaptive immunity and act to minimise immunopathology (196) while Th3 cells play a role in oral tolerance and providing help for IgA production, a noninflammatory antibody isotype, at mucosal sites (197). Because these subsets are relatively newly described, little is known about the factors involved in their differentiation. Using mouse models, Tr1 cells have been shown to require IL-27 for differentiation. This acts by inducing the major Tr1 transcriptional regulator c-MAF, IL-21 and the costimulatory receptor inducible T-cell costimulator (ICOS) (198). However, this pathway has yet to be fully investigated in humans.

Conventionally, identification of Treg cells has involved the use of markers such as CD4, CD25 and FOXP3, but because FOXP3 is an intracellular marker, the detection of which requires permeabilisation of cells and therefore their destruction, a reliable surface marker to substitute for FOXP3 was required for more in-depth studies of this cell type. The CD127 marker has since been identified as a reliable surface marker of $FOXP3^+$ Treg cells, with the

level of $CD4^+CD25^+CD127^{lo}$ cells correlating with the level of $CD4^+$ $CD25^+$ $FOXP3^+$ cells (199).

1.2.3.6 T Helper 17 Cells

The Th17 lineage was first described in 2005 (200) and is characterised by the production of its signature cytokines IL-17A, IL-17F, IL-22 and IL-26, which are involved in the recruitment of neutrophils (201, 202). Th17 cells are particularly important for host defence against fungi and extracellular bacteria (203, 204).

The generation of Th17 cells from naïve T cells occurs in three stages. The initial differentiation step requires TGFβ, IL-6 and IL-1β (205-207); TGFβ is a cytokine critical for iTreg differentiation because it promotes expression of FOXP3 (157), and it only results in Th17 differentiation when in low concentrations and in the presence of IL-6 (206). If TGFβ is present in high concentrations it can cause a change from Th17 to iTreg phenotype (157). The combination of TGFβ and IL-6 results in the activation of RAR-related orphan receptor (ROR)γt, the main transcriptional regulator of Th17 cells (205). In addition to RORγt, STAT3 has also been shown to be crucial for Th17 development because it induces the expression of RORγt (208). RORα was also found to be crucial in Th17 development because it works in close collaboration with RORγt. Absence of both RORγt and RORα results in complete loss of Th17 production (209).

After this initial differentiation stage of Th17 cells, IL-21 is involved in a self-amplification process whereby, in collaboration with TGFβ, a positive feedback loop is created to generate further Th17 cells (210). The final stage of differentiation involves the production of IL-23, which is not involved at all in the differentiation of these cells but is required purely for their survival and expansion (205).

1.2.3.7 Treg and Th17 Cells in Disease

Treg and Th17 cells are known to have an inverse relationship, with Th17 cells having proinflammatory properties and Treg cells being anti-inflammatory. Their developmental pathways are interrelated and evidence suggests that there is plasticity between the subsets (211); Treg and Th17 subsets have shown to be more susceptible to transformation than other $CD4^+$ subsets (212). For example, IL-6, a cytokine essential for Th17 differentiation, is able to convert a cell from Treg to Th17 phenotype (212), while Smad2 and Smad3, transcription factors essential for Treg differentiation, are able to hinder Th17 differentiation through inhibition of RORγt (213). This suggests that when considering the role of these subsets in

disease, analysing the balance between the two rather than each subset in isolation may be important. While there is little research on the balance between these two subsets in CF, other diseases may provide insight. In asthma a skewed balance between Treg and Th17 cells with dominance of the Th17 phenotype has been shown to contribute to disease pathology (214), and a similar effect of a predominance of the Th17 phenotype has been reported in inflammatory bowel disease (215).

Research has also shown there to be a link between the severity of inflammatory diseases and the level of IL-17 found in serum and tissue fluids of patients (200, 207). In particular, a study by Tiringer and colleagues (216) has shown the levels of IL-17 in the lungs of people with CF to be predictive of *P. aeruginosa* infection.

There is very little research investigating Tregs in CF and much of the research investigating Th17 cells in CF is focused on the lungs. The only study comparing levels of Treg cells in CF peripheral blood with those in healthy controls (217) showed that in CF, numbers of peripheral Tregs were decreased and their suppressive function was impaired. While it has been shown that Th17 cells predominate in CF lungs (216), a study by Tan and colleagues suggests that this could be a general feature of lung immunity (218). This group investigated the levels of IL-17 in the lungs of people with CF, non-CF bronchiectasis and healthy controls and showed that levels of Th17 cells were increased compared with controls in not only CF but also in non-CF bronchiectasis patients, although levels were higher in those with CF. Only two studies have compared peripheral blood Th17/IL-17 levels in CF with those in healthy controls (219, 220). One of these studies showed people with CF to have an increased percentage of Th17 cells and an increased propensity of naïve T cells to differentiate towards a Th17 phenotype (220). However, that study included only five CF participants, all of whom were female, and three healthy controls who were not age or sex matched, leading to considerable potential bias. The other study of Th17 cells in peripheral blood in fact found decreased IL-17 production by stimulated PBMC from people with CF (219). This study included a much larger, diverse participant group with an appropriately matched control population, providing much more reliable results. It did however show a link between high peripheral blood Th17 levels and reduced lung function. This may indicate that Th17 cells have homed to the lungs, where high levels have been reported and where their proinflammatory cytokines can be damaging, and that this is reflected in the periphery.

1.2.3.8 Relationship between $CD4^+$ T Cell Subsets

Several studies have recently shown that most $CD4^+$ T cell subsets are not terminally differentiated (Fig. 3), meaning that regulation of the balance of T cell subsets is even more vital than originally thought because a change in one subset may affect others. After exposure to IL-6, Treg cells have shown to alter their phenotype to that of Th17 (212), while Th17 cells can switch to Th1 following exposure to IL-12 (221) and to Th2 after exposure to IL-4 (222). Therefore, the balance between $CD4^+$ T cell subsets is tightly regulated, with Th1 and Th2 cells dominating over Th17 cells because IL-12, IFNγ and IL-4 inhibit Th17 differentiation.

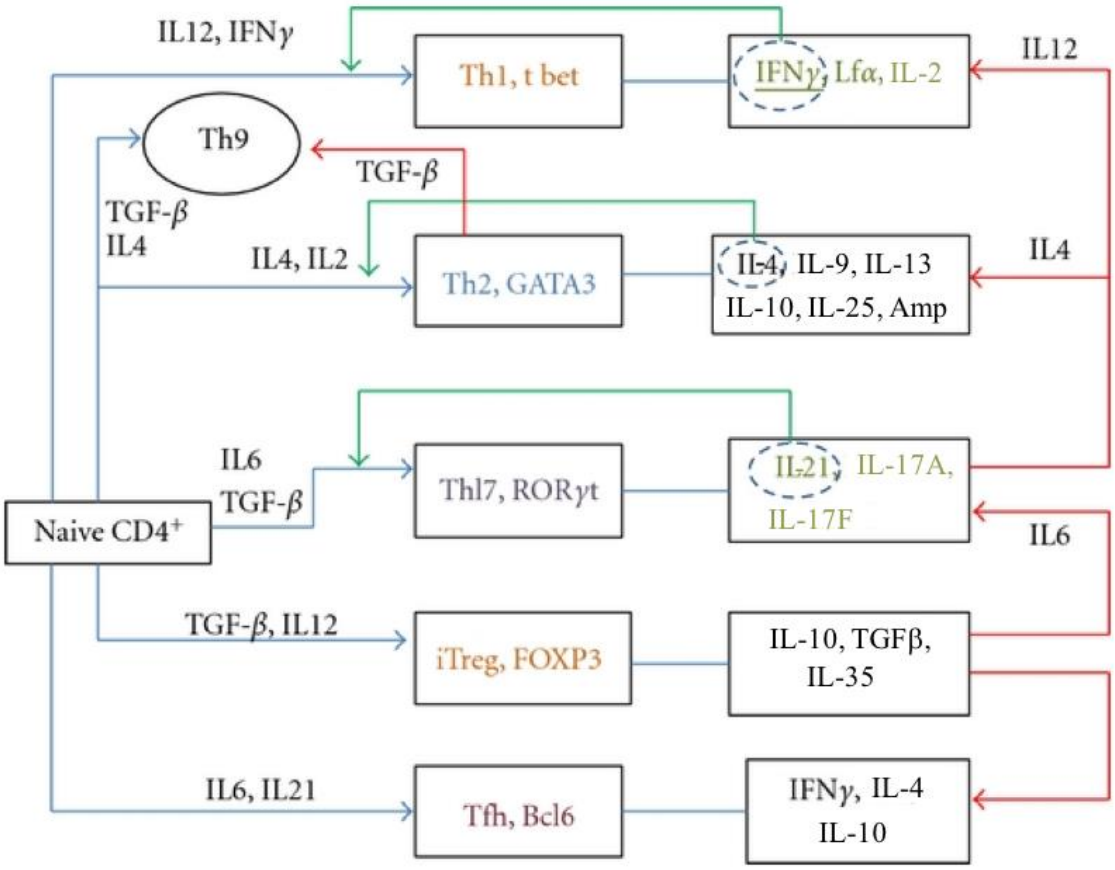


Fig. 3. The influence of various cytokines on the differentiation of CD4⁺ T cell subsets and expression of their major transcriptional regulators. Blue arrows represent the differentiation of naive T cells in the presence of specific cytokines. Green arrows represent the self-amplification loop directed by the circled cytokines. Red arrows represent the ability of subsets to alter their phenotype influenced by specific cytokines. Figure taken from Luckheerman *et al*. (154).

1.3.1 Homing of Immune Cells

The ability of immune cells, particularly lymphocytes, to migrate around the body is essential to their efficient and effective functioning. Lymphocytes must be able to home to the lymph nodes where antigen presentation takes place and towards sites of infection and inflammation in order to elicit an appropriate response. The primary mechanism of homing of immune cells involves chemokines and their binding to corresponding chemokine receptors on the surface of immune cells.

1.3.2 Chemokines

Chemokines are a family of chemotactic cytokines, approximately 8–12 kDa in size, that influence the homing of immune cells during both homeostasis and inflammation (223). They are divided into four subfamilies (CC, CXC, CX3C and XC) based on the structure and arrangement of their conserved cysteine residues (224). Homeostatic chemokines are involved in the maintenance of maturation, trafficking and regeneration of leukocytes under normal physiological conditions while inflammatory chemokines are involved in recruitment of leukocytes to inflamed/damaged tissues with production of these chemokines by both local tissues as well as local and infiltrating immune cells (225).

1.3.3 Chemokine Receptors and their Activation

Chemokine receptors are a group of transmembrane G-protein-coupled receptors (GPCRs) that can be responsive to multiple chemokines, although only to those within the same subfamily (226, 227). Activation of chemokine receptors not only controls leukocyte trafficking but also plays a role in cell degranulation, gene transcription, mitogenesis and apoptosis (228).

Binding of chemokines to these GPCRs results in the activation of phospholipase Cβ which cleaves itself to produce diacylglycerol (DAG) and inositol-1,4,5 triphosphate (IP3). DAG remains at the plasma membrane while IP3 is released into the cytosol to bind to the IP3 receptors on the endoplasmic reticulum (ER). This binding results in the release of Ca^{2+}, increasing cytosolic calcium concentrations. The increase in intracellular calcium results in localization of the membrane proteins calcium release-activated calcium channel protein 1 (ORAI1) and transient receptor potential cation channel, causing an influx of calcium through the plasma membrane. Calcium release-activated channels are also stimulated, further facilitating the influx of calcium. Further signalling events lead to actin polarisation and morphological changes in the responsive cell, leading to movement of that cell along the

chemokine concentration gradient towards an area of higher chemokine concentration (reviewed by Swaney *et al.* (229)).

In CF, defective CFTR, particularly the forms that are retained in the ER such as DF508, is known to cause increases in IP3-mediated intracellular calcium flux (230). It has also been shown to affect the plasma membrane localisation of ORAI1, resulting in an increased influx of calcium (231). Because calcium signalling is heavily involved in chemokine receptor activation and therefore leukocyte migration, this is a potential contributing factor to the dysregulated immune response in CF.

1.3.4 CXCR3, CCR4 and CCR6

Currently over 20 chemokine receptors and over 40 related chemokines have been identified (226). CXCR3 is a chemokine receptor primarily expressed on Th1 $CD4^+$ T cells, $CD8^+$ T cells and NK cells (232). It permits entry of these cells into sites of Th1-mediated inflammation, is activated by the IFNγ-induced chemokines CXCL9, CXCL10, and CXCL11 and has been implicated in many diseases states such as inflammatory bowel disease, leukaemia and diabetes (233).

CCR4 is a chemokine receptor predominantly expressed by Th2 cells, platelets and basophils and is responsive to the CCL17 and CCL22 chemokines (234). CCR4 has been implicated in many allergic diseases such as asthma (235).

CCR6 is a chemokine receptor, activated by CCL20, and is principally expressed by Th17 $CD4^+$ T cells but also $CD8^+$ T cells and immature DCs (236, 237). This chemokine receptor is unique in that it is activated by only one chemokine. Cells expressing CCR6 are able to home towards sites of Th17-mediated inflammation. CCR6 is an important chemokine receptor in mucosal immunity, allowing cells to enter the lungs and gut (238), and has been implicated in many inflammatory diseases such as rheumatoid arthritis (239).

1.3.5 Chemokines and Disease

While chemokines are beneficial for maintaining homeostasis and directing immune cells toward sites of infection and inflammation to promote an efficient and effective immune response, dysregulation of chemokine secretion by immune cells and tissues can contribute to disease pathology. Because chemokine receptors can be responsive to multiple chemokines (226, 227), understanding their role in disease states can be quite complex. Chemokine dysfunction has been implicated in many disease states such as autoimmunity, cancers, tumours, asthma, atherosclerosis, multiple sclerosis, arthritis, and infection (240).

In CF, two chemokines in particular, CXCL8 and CCL2, have been implicated in the pathogenesis of the disease (241-244). Excessive and prolonged production of these chemokines has been shown to promote recruitment to the lungs of inflammatory cells such as neutrophils and macrophages, which over time can become just as harmful to the lung as the infection they were recruited to combat.

1.4.1 The Immune System in Cystic Fibrosis

The role of the innate and adaptive immune systems in CF disease pathophysiology is still unclear, with many inconsistencies observed in immune responses to particular pathogens. While a significant amount of research has been devoted to exploring the effect of *CFTR* mutations in epithelial cells, a great deal of the CF phenotype cannot be explained simply by defective transport of ions by these cells. More recently, the intrinsic effects of mutated CFTR on the immune system have become of interest, because alteration of immune responses could exacerbate CF disease pathology and impede the ability of people with CF to clear pathogens effectively. Current knowledge about the dysregulation of immune cells in CF is summarised in Table 1.

1.4.2 Innate Immunity

It is currently accepted that the innate immune system in CF functions inappropriately on multiple levels. Studies have shown that there is a hyperinflammatory immune response to pathogens in CF, with an excessive production of proinflammatory cytokines (27). There are known dysfunctions of the innate immune system involving epithelial cells, extracellular matrix, pattern recognition receptors, and chemokine and cytokine secretion, as well as phagocyte recruitment and function (reviewed in (245)). More recently, innate immune cells such as neutrophils, macrophages and monocytes have become of interest because, as discussed in section 1.2.2, new findings suggest that they may express defective CFTR and that this could play a significant role in their ability to function appropriately. Overall, this evidence suggests that there are intrinsic defects in CF innate immune cells, although more research is required to determine the full effects and contribution of this to CF disease pathology. In general, there appears to be an exaggerated and prolonged immune response to pathogens in CF cells but a decreased ability to clear them.

1.4.3 Adaptive Immunity

More recently the adaptive immune system has become a topic of great interest in CF research, particularly because CFTR has been shown to be expressed by cells such as APCs

and T cells (21, 246), and because of the increasing complexity of the classification of $CD4^+$ T cell subsets. In addition to the characteristic hyperinflammatory immune response, people with CF also display a tendency to develop allergic reactions, asthma and dermatitis. In addition to this, they also display a characteristic Th2-biased immune response, particularly in response to infections with organisms such as *P. aeruginosa* (216, 247) and *A. fumigatus* (248), which has also been shown to become more dominant during pulmonary exacerbations (216). Th2-type immune responses favour allergies and are directed at clearing parasites rather than bacterial pathogens, which may partly explain why people with CF show ineffective clearance of pathogens such as *P. aeruginosa*.

Mueller and colleagues (22) have demonstrated that mutated CFTR in T cells results directly in abnormal immune responses. They showed that *Cftr*-knockout mice mounted an exaggerated IgE response to *A. fumigatus* infection compared with that of wild-type controls, and produced increased levels of IL-13 and IL-4, typical of a Th2-type response. A study by Moss and colleagues (20) has shown that activated CF lymphocytes produce lower levels of IFNγ (Th1 cytokine) and increased levels of IL-10 (Th2 cytokine). This has been suggested to downregulate the expression of costimulatory molecules on macrophages, which could affect antigen presentation and hence impair appropriate immune responses (108). A study by Moser and colleagues (249) has shown the importance of a Th1-type immune response in the effective clearance of pathogens, particularly *P. aeruginosa*. They demonstrated that when mice were repeatedly infected with *P. aeruginosa*, resistance developed concomitant with a shift to a Th1-dominated immune response and increased IL-12 production. There was no increase in serum IgG compared with mice that were infected only once, which indicates that a Th1-dominated cell-mediated immune response is protective against chronic *P. aeruginosa* infection in CF regardless of antibody production. Mueller and colleagues (22) demonstrated that in Cftr-deficient mouse T cells there was an increased intracellular Ca^{2+} flux in response to TCR-mediated activation. With this increased Ca^{2+} flux there was also an increase in nuclear localization of the calcium-sensitive transcription factor nuclear factor of activated T cells (NFAT). This was suggested to be responsible for the IL-13 response, because NFAT is a transcriptional regulator of cytokines directing Th2 responses (250). This study demonstrated how an alteration in the balance of Ca^{2+} can greatly affect gene expression in T cells. However, although the balance of the Th1/Th2 immune response is now known to be affected in CF, it is not known what other gene expression pathways are altered.

While the above evidence suggests intrinsic defects and alterations in the adaptive immune system in people with CF, it is not known to what degree this plays a part in CF disease pathology compared with alterations in the innate immune system and in epithelial cells. It is possible that the effects of CFTR on the immune system are responsible for significant disease pathology because of overall dysfunctional immune response.

Table 1. Current knowledge about innate and adaptive immune cell dysfunction in CF.

Cell Type	Alteration in CF	Reference
Neutrophils	Increased pulmonary levels	(100)
	Decreased microbicidal activity	(96-98)
	Reduced phagocytic capacity	(99)
	Increased elastase secretion	(251)
	Enhanced chemotaxis	(252)
	Reduced apoptosis	(253, 254)
	Altered cytokine production	(255)
Macrophages	Increased pulmonary levels	(102)
	Altered cytokine production	(256, 257)
	Reduced antigen presentation capacity	(258)
Monocytes	Reduced phagocytosis	(259)
	Increased elastase secretion	(90)
	Decreased adhesion	(85)
	Altered cytokine production	(88)
	Reduced antigen presentation capacity	(260)
Dendritic cells	Altered membrane structure	(23)
	Delayed differentiation	(23, 261)
	Decreased antigen presentation	(114)
Natural killer cells	Decreased peripheral blood levels	(138-140)
Myeloid derived suppressor cells	Unknown	
T Lymphocytes	Th2 & Th17 bias	(53, 262)
	Altered Cytokine production ($CD4^+$ and $CD8^+$)	(22, 115, 263)
B lymphocytes	Altered Immunoglobulin production	(145, 146, 149, 150)
	Altered differentiation	(144)

1.5.1 Hypothesis and Aims

We hypothesised that innate and adaptive immune dysfunction occurs in CF because of an intrinsic defect associated with mutated *CFTR* in immune cells and that this defect would be detectable as altered proportions of innate and adaptive peripheral blood immune cells. We aimed to characterise the proportions of $CD4^+$ effector (Th1, Th2 and Th17) and regulatory ($FOXP3^+$ Treg, IL-10^+ Tr1 and TGFβ^+ Th3) T cell subsets as well as the proportion of naïve and memory $CD4^+$ and Treg cells expressing the Th1-, Th2- and Th17-associated homing markers CXCR3, CCR4 and CCR6, respectively. In addition to this we aimed to determine the proportions of peripheral blood innate immune cells including monocytes, DCs, NK cells and MDSC and their subsets. We then aimed to investigate the gene expression of $CD4^+$ T cell subset-specific transcriptional regulators, chemokine receptors and inflammatory markers in $CD4^+$ T cells. Lastly, we aimed to determine the levels of all of these immune parameters in *CFTR* heterozygotes/carriers, who are clinically normal, to assess the degree to which defects are *CFTR*-mediated rather than infection- and inflammation-mediated.

CHAPTER 2

Methods

2.1 Study Participants and Blood Collection

Peripheral blood samples were obtained from CF patients (Ethics approval numbers H008013 and H0012530, Tasmanian Health and Medical Human Research Ethics Committee) attending the Royal Hobart Hospital. Participants included 22 children (0–17 years) and 20 adults (18–54 years) who were either attending outpatient clinics or were admitted because of clinical exacerbations. Clinical exacerbation was defined according to the Tasmanian Department of Health and Human Services' microbial surveillance and antimicrobial management guidelines (264), which define clinical exacerbation as the decline in two or more symptoms and one or more objective measures. Symptoms include cough, sputum volume, sputum purulence, self-reported breathlessness on exercise, fatigue and appetite. Objective measures include a reduction in absolute FEV1 of greater than 10% compared with the best achieved in the previous one year when stable, weight loss of greater than or equal to 1 kg in the past two weeks, increased respiratory rate at rest and new radiological changes on chest X-ray. Overall numbers of CF samples were limited by the number of patients in the Hobart region, but to obtain sufficient cells to allow full analysis of all patients, repeated samples were obtained over four years where possible, as indicated in Table 24-25. Before collection, informed consent was obtained either from patients themselves, or for children, from their legal guardians.

Peripheral blood samples for use as controls were obtained from healthy children (6 months–17 years) and adults (19–61 years) who had no history of chronic illness, no known family history of CF and no infectious illness within the previous two weeks. Informed consent was obtained from the adult volunteers, or from the legal guardians of the children.

Peripheral blood samples were also obtained from adult (22–47 years) CF heterozygotes (CF carriers), who were primarily the parents of the paediatric CF patients, following informed consent. The genotype of their defective *CFTR* allele was obtained where available.

Power calculations were performed based on our preliminary data of the immune cells of interest in CF and this suggested that, with an $\alpha = 0.05$ (95% confidence), samples sizes ranging from 10-40 would be sufficient. Sufficient participant numbers were achieved for each analysis.

Blood samples were collected in lithium heparin Vacuettes (Life Technologies, Mulgrave, VIC, Australia) and processed on the day of collection.

A detailed summary of the characteristics of all participants, including age, sex, type of infection and antibiotic treatment regime, clinical status, white cell count, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) inflammatory markers and lung function (as FEV1 % predicted) is given in Appendix 2.

2.2 Peripheral Blood Mononuclear Cell (PBMC) Isolation and Activation

Peripheral blood mononuclear cells (PBMC) were isolated using the density gradient medium Histopaque-1077 (Sigma Aldrich, Castle Hill, NSW, Australia) according to the manufacturer's instructions, then washed twice in phosphate-buffered saline (PBS) containing 10% foetal calf serum (FCS, Bovogen, East Keilor, VIC, Australia) at 300 × *g* for 10 min at room temperature. PBMC were either used fresh or were cryopreserved for future analysis. No difference was found in the cytokine expression of fresh versus cryopreserved cells: an example of flow cytometric staining of a control sample when fresh and then again after three months of cryopreservation is shown below (Figure 4). PBMC (2×10^6 cells) were activated in 4 mL of complete culture media (CCM) (detailed in Appendix 1a) in a 6-well tissue culture dish (Sigma-Aldrich, Castle Hill, NSW, Australia) at 37 °C in 5% CO_2 in air. Cells were activated for 5 h with 50 ng/mL phorbol myristate acetate (PMA) (Sigma Aldrich) and 1 µl/mL ionomycin (Life Technologies, Mulgrave, VIC, Australia). These were used as they stimulate the cells in a T cell receptor independent fashion. At the time of activation, 2.67 µL of BD GolgiStop was added to inhibit cytokine secretion.

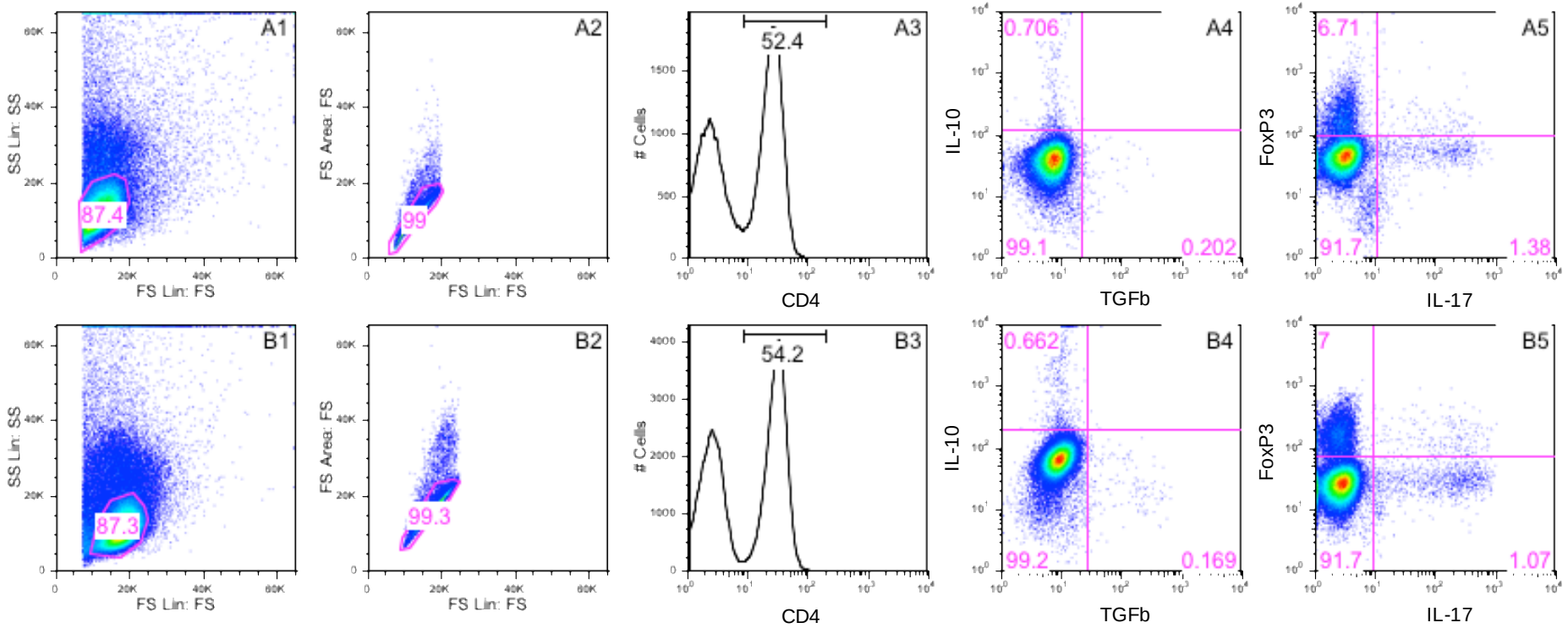


Fig. 4. Histograms demonstrating staining of the same PBMC sample when fresh (A1–A5) and after three months of cryopreservation (B1–B5).

'A1' and 'B1' show lymphocytes as a percent of total cells, 'A2' and 'B2' show single cells as a percent of lymphocytes, 'A3' and 'B3' show CD4 cells as a percent of lymphocytes, 'A4' and 'B4' show the percent of CD4 cells that are positive for IL-10 and TGF-β and 'A5' and 'B5' show the percent of CD4 cells that are positive for FOXP3 and IL-17A.

2.3 Cryopreservation of PBMC

PBMC were stored at 2–4 × 10^6 cells/mL in complete culture medium containing 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, Castle Hill, NSW, Australia). Freezing medium (CCM containing 10% DMSO) was prepared and cooled on ice. Cells were resuspended in ice-cold CCM then the freezing medium was added dropwise to the cells to minimize cell damage caused by the DMSO. Cells were transferred to cryovials and placed in a cooler box (Life Technologies, Mulgrave, VIC, Australia) at –80 °C. Within 3 days, cryovials containing cells were transferred into liquid nitrogen for longer-term storage.

2.4 Resuscitation of PBMC from Cryopreservation

Cells were removed from liquid nitrogen storage on ice and resuscitated immediately. CCM was heated in a water bath to 37 °C prior to retrieval of the cells. Cryovials were gently agitated in the 37 °C water bath until the medium had melted, then were gently transferred to a 15 mL tube (ThermoFisher Scientific, Scoresby, VIC, Australia). Two millilitres of warmed CCM was added dropwise to the cells, and then tube slowly topped up to 10 mL. Cells were then centrifuged at 500 × *g* for 5 min and resuspended in 1 mL of CCM for counting using a haemocytometer.

2.5 Immune cell phenotyping

Innate and adaptive peripheral blood immune cells were identified by flow cytometric analysis using the monoclonal antibodies listed in Table 2. Specific staining procedures for each antibody panel can be found in the methodology of chapters 3-5.

Table 2. Antibodies used for identification of innate and adaptive peripheral blood immune cell subsets

Antibody	Marker	Fluorochrome	Hybridoma Clone	Supplier
anti-CD4	$CD4^+$ helper T cells	Peridinin chlorophyll protein cyanin 5.5 (PerCP-Cy5.5)	SK3	BD Biosciences
anti-IFNγ	Th1 cells	Fluorescein isothiocyanate (FITC)	B27	BD Biosciences
anti-IL-4	Th2 cells	Allophycocyanin (APC)	MP4-25D2	BD Biosciences
anti-IL-17A	Th17 cells	Phycoerythrin (PE)	N49-653	BD Biosciences
anti-FOXP3	Treg cells	Alexa Fluor 647 (AF647)	259D	BD Biosciences
anti-IL-10	Tr1 cells	Allophycocyanin (APC)	JES3-19F1	BD Biosciences
anti-TGFβ	Th3 cells	Phycoerythrin (PE)	TW4	BD Biosciences
APC isotype control	rat IgG2a kappa	Allophycocyanin (APC)	R35-95	BD Biosciences
PE isotype control	mouse IgG1 kappa	Phycoerythrin (PE)	MOPC-21	BD Biosciences
Live/dead	Dead cells	Near infrared (IR)	–	ThermoFisher Scientific
anti-CD4	$CD4^+$ helper T cells	V500	RPA-T4	BD Biosciences
anti-CD25	Treg cells	Allophycocyanin (APC)	2A3-9E7	BD Biosciences
anti-CD127	Treg cells	Brilliant violet 421 (BV421)	A019D5	BioLegend
anti-CD45RO	Memory/naïve cells	BUV395	UCHL1	BD Biosciences
anti-CXCR3	Th1-associated cells	Alexa Fluor 488 (AF488)	1C6	BD Biosciences
anti-CCR4	Th2-associated cells	Phycoerythrin cyanin (PE-Cy7)	L291H4	BD Biosciences
anti-CCR6	Th17-associated cells	Phycoerythrin (PE)	11A9	BioLegend
anti-CD3	T cells	Biotin	OKT3	Centenary Institute, Newtown, NSW, Aus
anti-avidin	Biotin labelled cells (T and B cells)	Pacific orange (PO)	–	Centenary Institute
anti-CD19	B cells	Biotin	HIB19	BD Biosciences
anti-CD14	Monocytes Myeloid derived suppressor cells	Pacific Blue (PB)	M5E2	BD Biosciences
anti-CX3CR1	Monocytes Dendritic Cells	Fluorescein isothiocyanate (FITC)	2A9-1	BioLegend
anti-NKP46	Natural killer cells	Phycoerythrin (PE)	9E2	BD Biosciences
anti-CD16	Natural killer cells Monocytes Myeloid derived suppressor cells	Peridinin chlorophyll protein cyanin 5.5 (PerCP-Cy5.5)	3G8	BD Biosciences
anti-CD11b	Myeloid derived suppressor cells	Phycoerythrin cyanin (PE-Cy7)	ICRF44	BD Biosciences
anti-CD56	Natural killer cells	Allophycocyanin (APC)	NCAM16.2	BD Biosciences

2.6 Spirometry

Using a spirometer, forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1) values were recorded. FEV1 % predicted values were calculated according to the Australian CF Data Registry guidelines (265) to obtain lung function values that accounted for body mass, because both children and adults were included in the analyses.

2.7 Statistical Analysis

2.7.1 GraphPad Prism

All flow cytometric and gene expression data were analysed using GraphPad Prism Version 6 (GraphPad Software, La Jolla, CA). Because many parameters were not normally distributed, nonparametric tests (Mann-Whitney *U* and Kruskal–Wallis) were applied to the data to investigate the differences between groups. To determine correlations between clinical parameters and subset percentages a Spearman correlation was performed. All P values < 0.05 were considered significant.

2.7.2 Multi Experiment Viewer (MeV)

Raw data from experiments investigating immune cell subsets were imported into MultiExperiment Viewer (MeV) Version 4.8 (TM4 Microarray Software Suite, Boston, MA, USA) in the format of a .txt file. A heat map was automatically generated to allow visualisation of the relative expression of a marker by use of colour, with black indicating no change in expression, green indicating a downregulation in expression and red an upregulation in expression, all relative to the median of all samples combined.

Data were normalised across rows and an unsupervised clustering analysis was performed (hierarchical clustering). Hierarchical clustering was performed using a nonparametric Spearman rank correlation distance metric and average linkage clustering with an associated heat map expression image to visualise the grouping of parameters.

A nonparametric Kruskal–Wallis test was also performed using MeV to determine differences in the proportions of each subset between groups, and again a hierarchical clustering was performed and a heat map expression image created to visualise results.

2.7.3 Analyses within the CF Cohort

The relationships between peripheral immune cell percentages and clinical continuous variables such as ‘age’ and ‘FEV1 % predicted’ were assessed using a Spearman correlation.

Differences in peripheral immune cell percentages between subgroups of patients were also investigated. Patients were stratified by sex, clinically stable or exacerbating, those with or without chronic *P. aeruginosa* infection (defined by sputum culture on two or more occasions within a six month period), with or without current *S. aureus* infection, with or without current *A. fumigatus* infection (both assessed by sputum microbiology), with or without current treatment with any antimicrobial and genotype (DF508 homozygous, DF508 heterozygous, other). Genotypes were classified into these two groups as there were only two patients that did not possess a DF508 allele.

CHAPTER 3

Flow Cytometric Analysis of Peripheral Blood $CD4^+$ T Cell Subset Proportions

3.1 Introduction

The development of chronic infection and inflammation of the lungs is characteristic of CF, with *P. aeruginosa* being the most significant pathogen with respect to deterioration of lung function and reduced life expectancy. The inflammation present in the lungs is exaggerated, damaging, and ineffective for clearance of pathogens (266). A disruption in the balance of T cell responses in CF lung has been reported, with many studies observing a skewing towards a Th2- and Th17- dominated immune response (17, 22, 216, 248, 262, 263, 267, 268). However, whether this imbalance is specific to CF or is characteristic of inflammatory lung disease in general is still uncertain, given that similar features have been reported in other immunoinflammatory lung diseases such as asthma (269-272) and chronic obstructive pulmonary disease (COPD) (273-275).

Although *CFTR* encodes a chloride channel, this defective channel affects signalling through other ion channels including those specific for Ca^{2+} (276) and Na^{+} (277). An initial step in the activation of T cells is the influx of Ca^{2+} across the T cell membrane. The strength of this Ca^{2+} signalling, in part, influences the development of the naïve T cell into its functional subsets (e.g., Th1, Th2, Th17). Mueller and colleagues (22) have shown that in CFTR-deficient lymphocytes, enhanced intracellular Ca^{2+} influx leads to abnormal immune responses. While there are no studies involving Ca^{2+} signalling in human CF T cells, this is much evidence to show it is altered in other cells types (276) including human macrophages (278).

We therefore hypothesised that altered ion transport across the T cell membrane during stimulation would result in alterations in the relative proportions of peripheral $CD4^{+}$ T cell subsets. Shifts in proportions of cells such as Treg (279) and Th17 (268) have previously been reported in CF, as have alterations in peripheral cytokine concentrations (150), but it is not yet clear to what extent this is related to the CFTR defect or to the effects of chronic infection.

The proportions of three effector (Th1, Th2, Th17) and three regulatory ($FOXP3^{+}$ Treg, IL-10^{+} Tr1 and TGF-β^{+} Th3) $CD4^{+}$ subsets were measured in the peripheral blood of people with CF and compared with those in age-matched healthy controls, after stimulation with PMA and ionomycin. The use of ionomycin would allow the stimulation of these calcium pathways and as a result the detection of the signature intracellular cytokines of interest. The CF group was also further analysed to determine any correlations between subset proportions and relevant clinical variables.

3.2 Materials and Methods

3.2.1 Th1, Th2 and Th17 Staining of PBMC

Two million PBMC were activated as detailed in Chapter 2. After activation they were washed with FACS buffer and collected by centrifugation, 250 × *g* for 10 min. Cells were fixed using 1 mL of BD Cytofix Fixation buffer (BD Biosciences, San Jose, CA) for 15 min then washed a further two times with FACS buffer, 250 × *g* for 10 min. Cells were then permeabilised for 15 min using 1 mL of BD Perm/Wash buffer (BD Biosciences). The antibody combinations used for identification of Th1, Th2 and Th17 cells are listed in Table 3. Antibodies were incubated with cells for 30 min at room temperature protected from light. Cells were then washed twice with FACS buffer at 500 × *g* for 5 min, before being filtered through a 50 μm filter mesh in FACS buffer, to prevent cell clumping and blockages of flow cytometer fluidics, prior to flow cytometric analysis.

Table 3. Antibody combinations for identification of Th1, Th2 and Th17 subsets

Antibody	Marker	Fluorochrome	Hybridoma Clone	Supplier
anti-CD4*	$CD4^+$ helper T cells	Peridinin chlorophyll protein cyanin 5.5 (PerCP-Cy5.5)	SK3	BD Biosciences
anti-IFNγ*	Th1 cells	Fluorescein isothiocyanate (FITC)	B27	BD Biosciences
anti-IL-4*	Th2 cells	Allophycocyanin (APC)	MP4-25D2	BD Biosciences
anti-IL-17A*	Th17 cells	Phycoerythrin (PE)	N49-653	BD Biosciences

* These antibodies are components of a commercial cocktail (Human Th1/Th2/Th17 phenotyping cocktail Cat.No.560751)

3.2.2 Treg, Th17, Tr1 and Th3 Staining of PBMC

Two million PBMC were activated as detailed in Chapter 2. After activation they were centrifuged and washed with FACS buffer, 500 × *g* for 5 min. Cells were then fixed using 2 mL of a 1:10 dilution of FOXP3 fixation buffer A (BD Biosciences) for 15 min. After washing twice with FACS buffer at 500 × *g* for 5 min, cells were permeabilised using 0.5 mL of FOXP3 permeabilisation buffer C (BD Biosciences) for 30 min. Cells were again washed twice with FACS buffer at 500 × *g* for 5 min. The antibody combinations used for identification of Treg, Th17, Tr1 and Th3 subsets are listed in Table 4. Antibodies were incubated with cells for 40 min at room temperature protected from light. Cells were then washed twice with FACS buffer at 500 × *g* for 5 min, before being filtered through a 50 μm filter mesh in FACS buffer prior to flow cytometric analysis.

Table 4. Antibody combinations for identification of Treg, Th17, Tr1 and Th3 subsets

Antibody	Marker	Fluorochrome	Hybridoma Clone	Supplier
anti-CD4*	$CD4^+$ helper T cells	Peridinin chlorophyll protein cyanin 5.5 (PerCP-Cy5.5)	SK3	BD Biosciences
anti-IL-17A*	Th17 cells	Phycoerythrin (PE)	N49-653	BD Biosciences
anti-FOXP3*	Treg cells	Alexa Fluor 647 (AF647)	259D	BD Biosciences
anti-CD4	$CD4^+$ helper T cells	Peridinin chlorophyll protein cyanin 5.5 (PerCP-Cy5.5)	SK3	BD Biosciences
anti-IL-10	Tr1 cells	Allophycocyanin (APC)	JES3-19F1	BD Biosciences
anti-TGFβ	Th3 cells	Phycoerythrin (PE)	TW4	BD Biosciences
APC isotype control	rat IgG2a kappa	Allophycocyanin (APC)	R35-95	BD Biosciences
PE isotype control	mouse IgG1 kappa	Phycoerythrin (PE)	MOPC-21	BD Biosciences

* These antibodies are components of a commercial cocktail (Human Treg/Th17 phenotyping cocktail Cat.No.560762)

3.2.3 Flow Cytometric Data Acquisition and Analysis

Cells were analysed on a CyAn ADP 9 colour flow cytometer (Beckman Coulter, Lane Cove, NSW, Australia) with blue 488 nm, violet 405 nm and red 633 nm lasers allowing measurement of nine fluorescence and two scatter parameters using Summit software (Beckman Coulter). Data obtained from the flow cytometer were analysed using FlowJo software (Treestar, Ashland, OR). Figure 6 demonstrates the hierarchical gating strategy used to identify the $CD4^+$ subsets. Unstimulated controls were run for self-designed panels (CD4, TGFβ, IL-10), an example of which is shown in Figure 5, to detect non-specific staining.

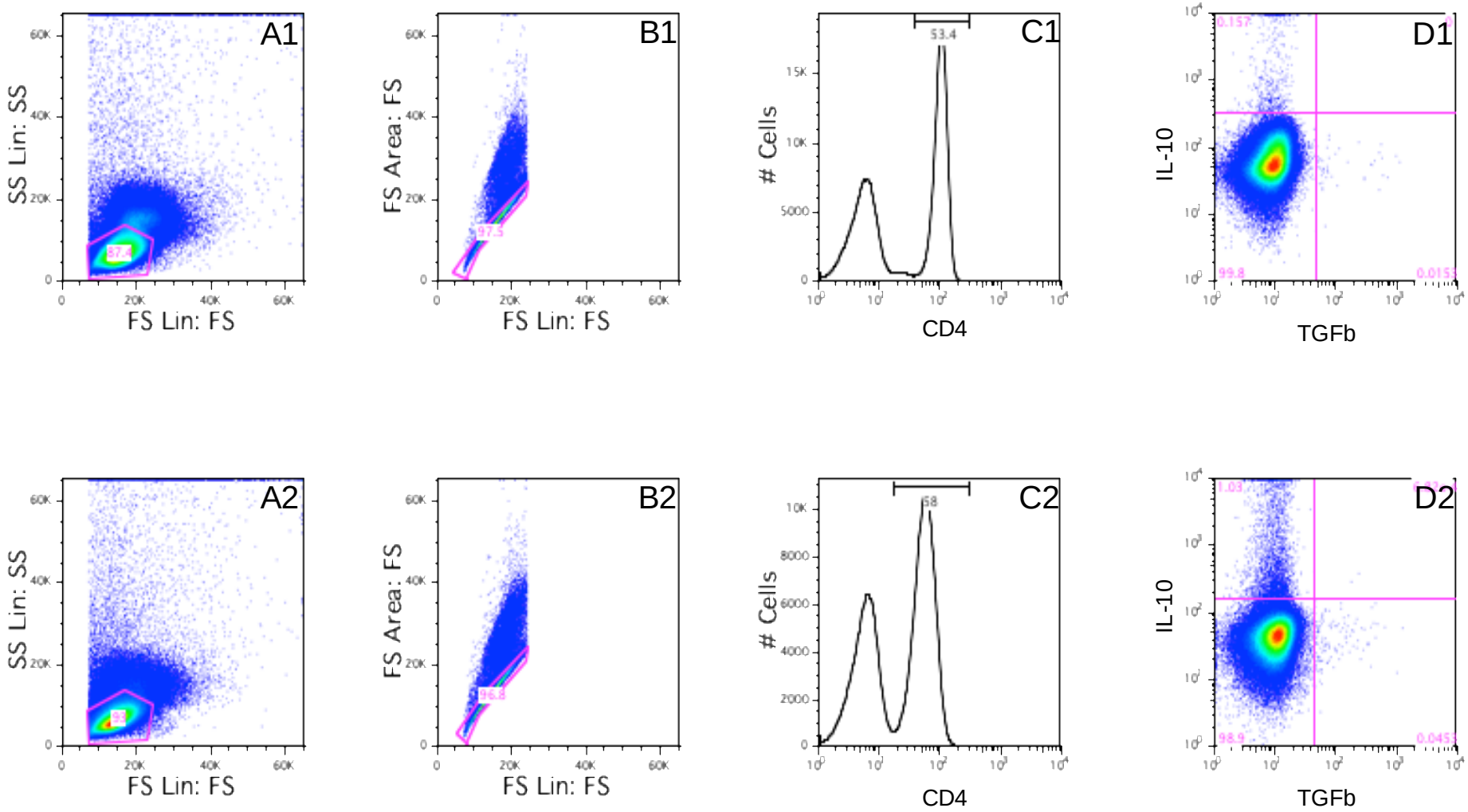


Fig. 5. Representative histograms from the same healthy individual demonstrating unstimulated cells compared with stimulated cells for the same staining procedure to identify non-specific labelling. 'A1 & A2' shows lymphocytes as a percent of total cells, 'B1 & B2' shows single cells as a percent of lymphocytes, 'C1 & C2' shows $CD4^+$ cells as a percent of lymphocytes, and 'D1 & D2' shows the percent of $CD4^+$ cells that are positive for IL-10 and TGFβ.

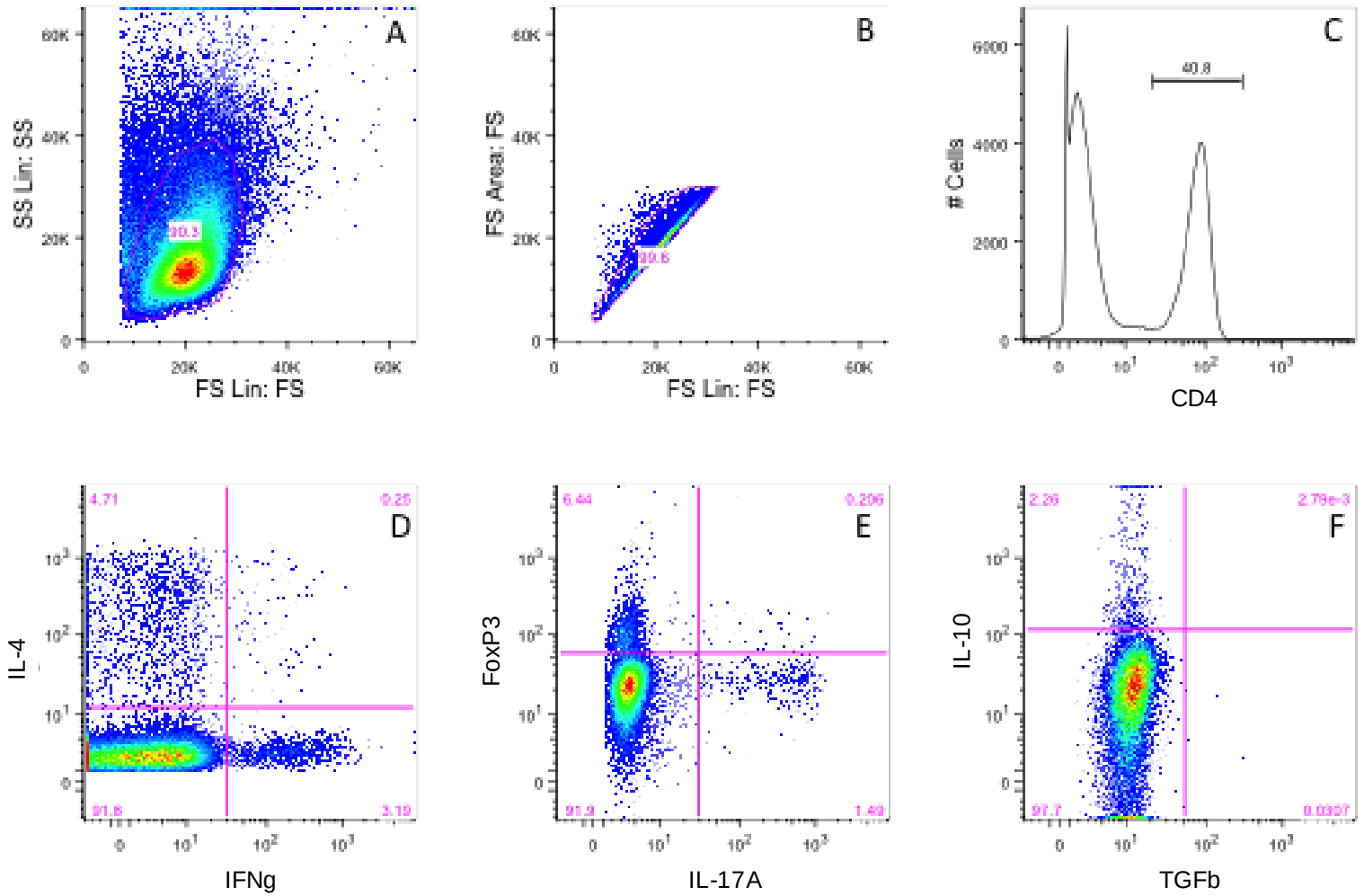


Fig. 6. Representative histograms from healthy individual demonstrating the gating strategy for flow cytometry analysis of CD4$^+$ T cell subsets. 'A' shows lymphocytes as a percent of total cells, 'B' shows single cells as a percent of these lymphocytes, 'C' shows CD4$^+$ cells as a percent of these singlet lymphocytes, and 'D','E' and 'F' show the percent of these CD4$^+$ cells that are positive for IL-4, IFNγ, FOXP3, IL-17A, IL-10 and TGFβ.

3.3 Results

3.3.1 Comparison between CF and Control Groups

Median age, sex and $CD4^+$ subset percentages of the CF and control groups are detailed in Table 5. Subset percentages for healthy controls corresponded with that published within the literature (280). No significant differences between the CF and control groups were found for median age, sex distribution, or the proportions of $CD4^+$ effector subsets (Th1, Th2, Th17). Proportions of all the $CD4^+$ regulatory subsets were found to be elevated in the CF group compared with the control group (*p* values for $FOXP3^+$ Treg% = 0.015, IL-10^+ Tr1% = 0.0001, TGFβ^+ Th3% < 0.0001).

To explore further the role of infection versus that of an underlying CFTR defect in the increases in $CD4^+$ subsets, the groups were subdivided into adults and children for analysis (Table 6). All adults had multiple lung infections and all but two children were free of any culture-detectable infection. The increases in regulatory subsets were found to be present only in the adult CF group, while the levels in CF children did not differ significantly from those in control children.

Because of the many reports of Th2- and Th17-skewed immune responses in CF, the Th1/Th2 and Treg/Th17 ratios were calculated (Tables 5 and 6). There was no significant difference in the Th1/Th2 ratio between the control and CF groups. The Treg/Th17 ratio was much more variable in the CF group and was trending towards being higher than in controls (*P* = 0.066) which is consistent with the observed increase in proportion of Tregs in the CF group.

Table 5. Comparison of $CD4^+$ T cell subset percentages between control and CF groups

	Control	Cystic Fibrosis	*P* Value
N	68	42	
Age median (range)	21 (2–61)	16 (0.5–53)	0.15
Sex male (%)	33 (48%)	20 (47%)	
$CD4^+$%# median (range)	48.8 (29.7–66.0)	47.0 (28.3–66.1)	0.60
Th1%* median (range)	7.9 (3.0–22.4)	6.27 (1.2–24.6)	**0.04**
Th2%* median (range)	2.3 (0.4–9.8)	1.7 (0.35–8.5)	0.09
Th1/Th2 ratio* median (range)	4.5 (1.3–11.3)	4.8 (0.7–10.4)	0.81
Th17%* median (range)	0.47 (0.08–2.26)	0.43 (0.01–1.98)	0.37
Treg%* median (range)	5.8 (1.8–11.2)	7.1 (2.5–12.8)	**0.016**
Treg/Th17 ratio* median (range)	11.9 (2–57)	16.2 (3.2–441)	0.066
IL-10^+ Tr1%* median (range)	1.18 (0.35–3.32)	1.63 (0.41–4.16)	**0.0001**
TGFβ^+ Th3%* median (range)	0.027 (0.008–0.190)	0.055 (0.017–0.417)	< **0.0001**

P values for Mann–Whitney *U*–test are shown; significant differences are shown in **bold.**
* Indicates all values are calculated as a percentage of the parent population.
# Indicates all values are calculated as a percentage of the parent population.

Table 6. Comparison of CD4+ T cell subset percentages between control and CF in adults and children

	ADULTS			CHILDREN		
	Control	**Cystic Fibrosis**	***P* value**	**Control**	**Cystic Fibrosis**	***P* value**
N	51	20		17	22	
Age median (range)	22 (18–61)	24 (18–53)	0.13	8 (2–17)	11 (0.5–17)	0.22
Sex male (%)	24 (47%)	12 (60%)		9 (51%)	8 (36%)	
$CD4^+$%# median (range)	50.40 (29.7–66.0)	48.15 (28.3–66.1)	0.18	42.1 (32.7–52.6)	46.8 (33.2–61)	0.15
Th1%* median (range)	9.6 (3.0–22.4)	10.45 (3.5–24.6)	0.82	4.6 (3.0–9.6)	5.4 (1.2–10.7)	0.98
Th2%* median (range)	2.6 (0.7–9.8)	2.3 (0.4–8.5)	0.74	1.4 (0.4–5.0)	1.2 (0.4–8.4)	> 0.99
Th1/Th2 ratio* median (range)	4.6 (1.3–11.3)	5.1 (1.0–10.4)	0.67	3.8 (1.3–8.5)	3.9 (0.7–8.8)	0.91
Th17%* median (range)	0.49 (0.15–2.26)	0.52 (0.04–1.98)	0.77	0.37 (0.08–1.08)	0.36 (0.03–1.02)	0.79
Treg%* median (range)	5.5 (1.8–10.5)	6.46 (4.2–11.4)	**0.01**	7.6 (3.5–11.2)	7.2 (2.5–12.8)	0.77
Treg/Th17 ratio* median (range)	10.6 (2–37)	14.8 (4–441)	0.32	18.6 (6.9–57)	18.6 (3.2–305)	0.93
IL-10^+ Tr1%* median (range)	1.15 (0.35–2.63)	1.77 (0.41–4.16)	**0.0004**	1.27 (0.70–3.32)	1.5 (0.66–2.85)	0.29
TGFβ$^+$ Th3%* median (range)	0.022 (0.008–0.19)	0.093 (0.018–0.417)	< **0.0001**	0.044 (0.020–0.143)	0.043 (0.017–0.301)	0.86

P values (Mann–Whitney *U* test) of significant differences are shown in **bold.**
* Indicates all values are calculated as a percentage of the parent population.
# Indicates all values are calculated as a percentage of the parent population.

3.3.2 Comparison within the CF Cohort

We next investigated any important associations between $CD4^+$ subset percentages and clinical parameters within the CF group. Table 7 details all parameters assessed.

Analysis of the *CFTR* genotype showed that all but three patients had at least one DF508 allele, therefore patients were divided into those who were DF508 homozygous (n = 18) and those who were DF508 heterozygous or had other genotypes (n = 24). No significant correlations were found based on this division of *CFTR* genotype.

To examine the effects of clinical status, the CF group was divided into those who were clinically stable (n = 35) and those who were exacerbating (n = 7). Exacerbating patients were defined according to the Tasmanian Department of Health and Human Services' microbial surveillance and antimicrobial management guidelines (264). No significant correlations were found based on clinical status.

For patients with chronic *P. aeruginosa* (n = 15) there was a significantly higher $TGF\beta^+$% (P = 0.0006) and a trend towards higher Th17% (P = 0.09) than in patients without chronic *P. aeruginosa* infection. The percentages of these cells were also both found to be significantly different (TGF-β^+% P = 0.05; Th17% P = 0.01) in patients on antimicrobial treatment (n = 21) compared with those not on antimicrobials. No differences were found between patients with and without other infections such as *S. aureus* and *A. fumigatus*.

Table 7. Correlations between CD4⁺ T cell subset percentages and clinical variables in the CF group.

Parameter [no. of patients]	CD4⁺%	Th1%	Th2%	Th17%	Treg%	Tr1%	Th3%	Th1/Th2	Treg/Th17
Age [42] (Spearman *r*)	–0.032	0.56	0.44	0.23	0.06	0.12	0.29	0.16	-0.11
***P* value**	[0.84]	**[0.002]**	**[0.018]**	[0.16]	[0.73]	[0.46]	[0.07]	[0.43]	[0.51]
FEV1 % pred. [32] (Spearman *r*)	–0.01	–0.45	–0.31	–0.56	–0.42	0.003	0.04	–0.25	–0.12
***P* value**	[0.95]	[0.06]	[0.19]	**[0.0008]**	**[0.016]**	[0.99]	[0.82]	[0.28]	[0.57]
FEV1 % pred. stable patients only [25] (Spearman *r*)	–0.13	–0.45	–0.31	–0.58	–0.31	0.16	0.16	–0.10	–0.14
***P* value**	[0.52]	[0.06]	[0.19]	**[0.00017]**	[0.12]	[0.44]	[0.44]	[0.68]	[0.52]
FEV1 % pred. *P. aeruginosa* negative, <18 only [6] (Spearman *r*)	0.43	0.00	–0.20	–0.83	0.09	–0.54	–0.03	–0.10	0.83
***P* value**	[0.40]	[1.00]	[0.75]	**[0.042]**	[0.87]	[0.27]	[0.96]	[0.87]	**[0.042]**
Median percent for each subset									
Sex									
Male	46.3	7.8	1.80	0.43	7.1	1.60	0.090	4.8	14.4
Female	48.2	5.7	1.42	0.40	6.9	1.67	0.055	4.7	17.0
***P* value**	[0.25]	[0.38]	[0.32]	[0.49]	[0.96]	[0.69]	[0.13]	[0.84]	[0.30]
Genotype									
DF508 homozygous [18]	47.8	5.7	1.55	0.41	6.7	1.76	0.053	4.9	13.3
DF508 heterozygous/other [24]	46.8	6.2	1.42	0.43	7.6	1.47	0069	4.8	19.5
***P* value**	[0.74]	[0.85]	[0.99]	[0.69]	[0.12]	[0.48]	[0.66]	[1.0]	[0.28]
Stable [35]	47.0	6.2	1.42	0.43	7.0	1.65	0.053	4.7	17.0
Exacerbating [7]	48.2	13.1	2.35	0.65	7.1	1.56	0.079	5.90	9.9
***P* value**	[0.94]	[0.26]	[0.32]	[0.14]	[0.60]	[0.99]	[0.76]	[0.13]	[0.18]
Chronic *P. aeruginosa* [15]	46.6	9.6	2.32	0.66	6.8	1.44	0.095	5.8	11.2
No chronic *P. aeruginosa* [27]	47.8	5.7	1.63	0.36	7.3	1.63	0.041	3.9	18.7
***P* value**	[0.42]	[0.17]	[0.42]	[0.09]	[1.0]	[0.69]	**[0.006]**	**[0.04]**	[0.18]
Current *S. aureus* infection [21]	48.1	5.7	2.11	0.42	6.8	1.77	0.044	4.8	16.6
No *S. aureus* infection [26]	46.5	6.4	1.42	0.51	7.1	1.63	0.088	4.7	13.8
***P* value**	[0.59]	[0.79]	[0.50]	[0.51]	[0.76]	[0.53]	[0.17]	[0.87]	[0.34]
Current *A. fumigatus* infection [7]	46.3	8.9	1.8	0.43	6.3	1.54	0.091	4.9	18.8
No *A. fumigatus* infection [35]	47.5	5.4	1.52	0.43	7.1	1.65	0.048	4.4	15.8
***P* value**	[0.42]	[0.06]	[0.36]	[0.92]	[0.48]	[0.75]	[0.25]	[0.56]	[0.97]
Any current antimicrobial [21]	47.6	8.5	2.0	0.71	6.8	1.76	0.092	5.2	9.3
No current antimicrobial [21]	46.6	5.7	1.69	0.34	7.1	1.54	0.041	3.7	18.8
***P* value**	[0.68]	[0.07]	[0.35]	**[0.013]**	[0.93]	[0.42]	**[0.047]**	[0.13]	**[0.02]**

[P values] (Mann–Whitney U test) indicating significant differences are shown in **bold**.

Analysis of the association between FEV1 % predicted and $CD4^+$ subset percentages was performed on a smaller subset of patients for whom lung function tests were available (n = 32, 17 adults, 14 children). This group had a slightly higher median age (19.5 years), contained no children aged under 5 years, and had a higher percentage of males (59%). All other variables were similar to the whole CF cohort. In this subgroup, a strong correlation was found between FEV1 % predicted and Th17% (r = –0.56, *P* = 0.0008, Spearman) as well as a significant correlation with Treg% (r = –0.42, *P* = 0.016, Spearman), with increasing levels of these subsets both associated with declining lung function (Fig. 7).

Because lung function testing was not always performed on the same day as blood sampling, and because lung function can change quite quickly with infections of the lung and the treatment of exacerbations, a more in-depth analysis of the relationship between Th17% and Treg% with FEV1 % predicted was performed focusing on clinically stable patients, so as to reduce the influence of acute infections.

Firstly, patients who were exacerbating on the day of blood sampling (n = 5) were excluded. When only clinically stable patients were analysed, the relationship between FEV1 % predicted and Th17% was still present (r = –0.58, *P* = 0.0017), but the association with Treg% was no longer significant (*P* = 0.11).

Secondly, because of reports of *P. aeruginosa*- and age-related T cell defects in CF, children (<18 y) without chronic *P. aeruginosa* infection (n = 6) were analysed. The relationship between FEV 1 % predicted and Th17 precent remained (r = –0.83, *P* = 0.042) while the association with Treg% did not (r = 0.09, *P* = 0.87).

Thirdly, the relative timing of blood sampling to lung function testing was considered in the analysis. When investigating this timing it was apparent that the correlation was strongest when the two tests were performed within a week of each other (Th17% r = –0.86, *P* = < 0.0001; Treg% r = –0.62, *P* = 0.01; n = 16) and was no longer significant when the two tests were performed more than two weeks apart.

The use of antimicrobials and the presence of chronic *P. aeruginosa* infection were also investigated in relation to these associations, however these analyses were compromised because patients on antimicrobials and with chronic *P. aeruginosa* infection were mostly adults and those without either were mostly children (median age on antimicrobials 22 years; median age without antimicrobials 13 years; median age with chronic *P. aeruginosa* infection 22 years; median age without chronic *P. aeruginosa* infection 11 years). Median lung function

was also therefore altered, with those patients not on antimicrobials and those without chronic *P. aeruginosa* infection having a high FEV1 % predicted (97% and 85% respectively) while for those on antimicrobials and those with chronic *P. aeruginosa* infection it was much lower (54% and 51% respectively). However, the relationship between FEV1 % predicted and Th17% remained significant in the group on antimicrobials ($P = 0.03$, n = 18) and trended towards significance in the group with chronic *P. aeruginosa* infection ($P = 0.056$, n = 15).

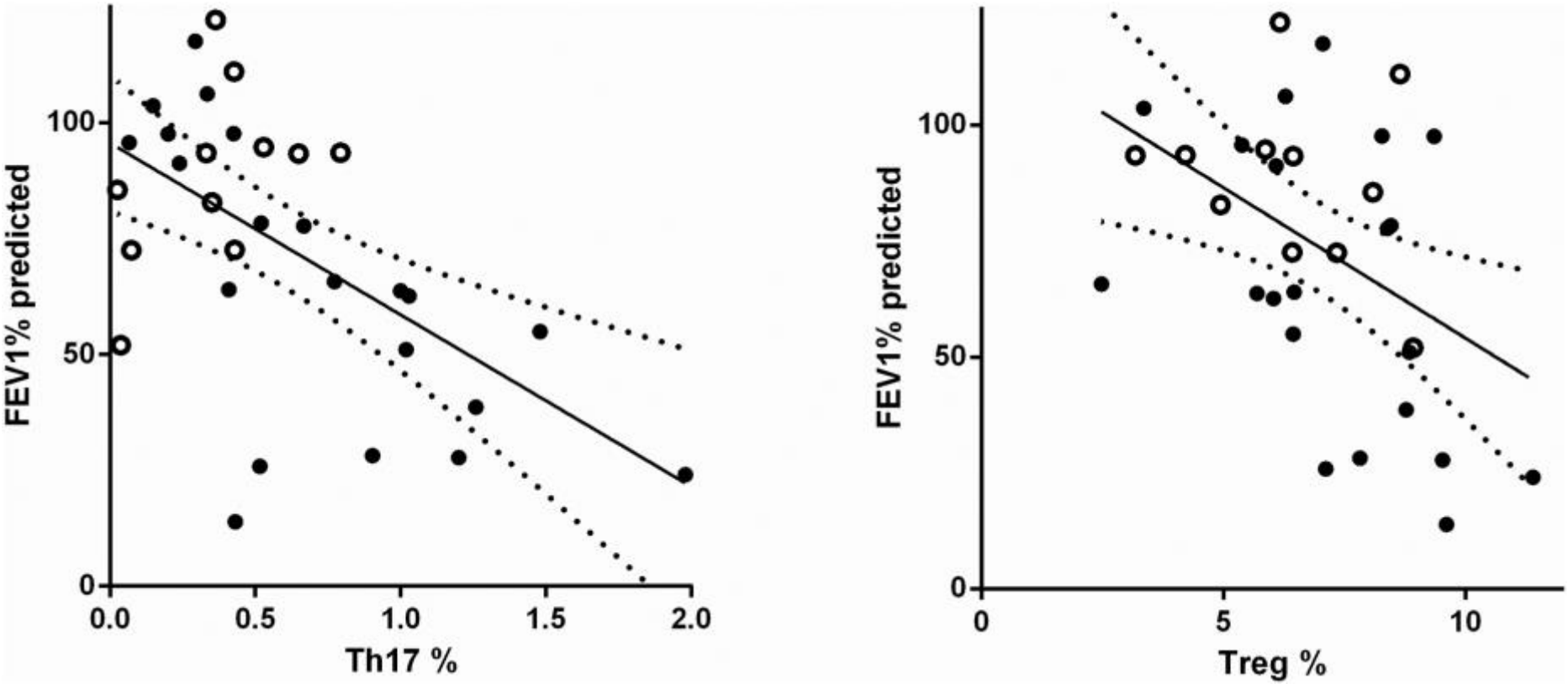


Fig. 7. Correlation of Th17% and Treg% with FEV1 % predicted in the CF group. Analysis was performed using a nonparametric Spearman correlation. The solid line indicates the linear regression line and the dotted lines indicate the 95% confidence intervals. Solid closed circles indicate those patients for whom blood testing and spirometry were performed within a 2-week period of each other (n = 21). Open circles indicate those patients for whom blood testing and spirometry were performed more than 2-weeks apart (n = 11).

Table 8. Correlations between Th17% and Treg% with FEV1 % predicted based on time elapsed between blood sampling and lung function testing in the CF group.

Group (no.)	Age years median (range)	% male	Th17% Spearman *r* (*P* value)*	Treg% Spearman *r* (*P* value)*
All (32)	19.5 (6–53)	59%	–0.57 **(0.0008)**	–0.42 **(0.016)**
FEV1 % predicted test on same day as blood sampling (10)	22 (10–53)	70%	–0.85 **(0.003)**	–0.60 (0.07)
FEV1 % predicted test within 1 week of blood sampling (16)	22 (10–53)	63%	–0.86 **(< 0.0001)**	–0.62 **(0.01)**
FEV1 % predicted test within 2 weeks of blood sampling (21)	20 (10–53)	67%	–0.72 **(0.0003)**	–0.49 **(0.02)**
FEV1 % predicted test more than 2 weeks from blood sampling (11)	11 (6–38)	45%	0.49 (0.12)	–0.35 (0.29)

P* values indicating significant differences are shown in **bold.

3.4 Discussion

Th17 cells have been implicated in many inflammatory lung diseases, but this is the first report of a link between lung function (FEV1 % predicted) and Th17% in the peripheral blood of CF patients, rather than that in the lung or bronchoalveolar lavage fluid (BALF). This relationship has been reported previously in COPD (275, 281), where the percent Th17 cells was also increased. A study by Xu *et al.* (282) of COPD peripheral blood also demonstrated a similar relationship of lung function with IL-17-producing $CD8^+$ (Tc17) cells.

Many studies have demonstrated the importance of Th17-mediated inflammation in CF lung pathology (53, 220, 283). Although there was no significant difference in peripheral blood Th17% between CF patients and healthy controls, the strong inverse relationship between Th17% and FEV1 % predicted within the CF group is consistent with these reports of the damaging effects of Th17 cells in the lungs. It is also quite remarkable that this strong negative correlation was observed in peripheral blood, away from the site of infection, rather than as previously reported in the lungs (284) and BALF (53). This association only became stronger the closer together spirometry and blood tests were performed. In CF adults a trend was seen (P = 0.09) towards an association between chronic *P. aeruginosa* infection and Th17%. It has been reported that Th17-dominated immune responses are a risk factor for *P. aeruginosa* infection, possibly because they are ineffective against this pathogen (216) as they recruit neutrophils that, in CF, are ineffective or because the antibody response elicited is also ineffective (285). This may explain the strong inverse correlation of peripheral blood Th17% with FEV1 % predicted, because those with a higher Th17% may be predisposed to this highly damaging lung infection. This theory is also consistent with the association seen between higher Th17% and treatment with antimicrobial therapy. However, because this was a cross-sectional study, causality could not be confirmed.

An association between FEV1 % predicted and Treg% was also observed for the whole CF group. However, when the group was divided into stable and exacerbating patients, this association was no longer significant. Treg cells have been shown to have a reduced suppressive capacity specifically during *P. aeruginosa* infection (217), and it may be that levels of these cells increase in those who are exacerbating in order to compensate for their reduced suppressive capacity. It is also quite curious to see that both Treg% and Th17% were negatively correlated with FEV1 % predicted, when these two subsets are often described as mutually antagonistic. Because Treg cells are anti-inflammatory and dampen effector immune

responses, it would be expected that a high Treg% would be associated with less inflammation and thus improved lung function. Other studies have shown a positive correlation between FEV1 % predicted and Treg cells and an *P. aeruginosa*-linked and age-dependent loss of Treg function, however these studies involved mainly infants and adolescents (217, 279). In our study, when lung function was investigated only in those children (< 18 years) without *P. aeruginosa* for whom lung function tests were available (n = 6), there was no longer a negative correlation between Treg% and FEV1 % predicted. The presence of the negative correlation between Treg% and FEV1 % predicted for the entire CF group may be the result of the reported reduced suppressive capacity of Treg cells during inflammation and *P. aeruginosa* infection (217) (286). This is further supported by the positive association observed between FEV1 % predicted and the Treg/Th17 ratio in the children without *P. aeruginosa* infection.

When considering the relationship between Th17% and FEV1 % predicted it should be noted that there was a negative correlation between FEV1 % predicted and age (Table 7). However, because there was no association between Th17% and age, it is likely that the link between Th17% and FEV1 % predicted is unrelated to age, although a multivariate analysis in a larger group is required to confirm this. The higher Th17% found in peripheral blood of patients with poor lung function may reflect a higher level of these cells in the lungs, at the main site of infection, which could be associated with lung damage (263). However, while there is no published information regarding the homing of Th17 cells in CF, the only two studies (219, 287) that have investigated Th17 in CF peripheral blood reported reduced levels of these cells. Given the many studies indicating high levels of lung Th17 in CF, we can speculate that this could be the result of homing of these cells to the lungs. The reason why our study did not show reduced levels of Th17 cells in the periphery may be related to our identification technique, because some $CD4^+$ populations other than Th17 cells, such as γδ T cells, are capable of producing IL-17.

It is also possible that as has been reported for high lung Th17% (53), a high peripheral blood Th17% may be a predisposing factor to chronic *P. aeruginosa* acquisition, which in turn is a cause of lung damage.

Lastly, a high Th17% could also be related to an individual's inherent propensity towards a Th17-biased immune response. If this propensity is similar to that for Th2-biased allergic responses, which are formed and detected at an early age (288, 289), Th17 cells could potentially be a useful prognostic indicator of susceptibility to *P. aeruginosa* acquisition and

therefore lung function decline. However, because the correlation between FEV1 % predicted and Th17% was strongest when spirometry and blood sampling were performed close together in time, it would appear that the Th17% changes may be synchronised with changes in lung function. Additional research would be required to determine any prognostic value for lung function decline of Th17% in peripheral blood.

Previous studies have reported a bias towards increased Th2 and Th17 cells in CF lung and peripheral blood (53, 220), but no such bias was found in this study, with no significant increase in peripheral blood percentages of either the Th2 or Th17 subsets, and no difference between CF and controls in the peripheral blood Th1/Th2 and Treg/Th17 ratios. This discrepancy may simply be due to more complex sets of identification markers used in other studies. However, there a was an significant decrease in Th1 percent in CF compared with healthy controls indicating a Th2 dominated response in CF.

A significant increase was found in the percentages of three regulatory subsets measured, $FOXP3^+$ Treg, IL-10^+ Tr1 and $TGF\beta^+$ Th3 cells, in CF adults (>18 years) compared with control adults. It was unexpected to see an increase in IL-10-producing cells, because many studies have previously reported a decrease of this cytokine in CF peripheral blood (290, 291). While there is very little information available regarding $TGF\beta^+$ Th3 cells in CF, reports of increased TGFβ in plasma (292), sputum (293) and BALF (294) in CF and in one study of peripheral blood $CD4^+$ cells of *A. fumigatus*-infected CF patients (51) are consistent with this finding.

The increase in $FOXP3^+$ Treg% barely reached significance compared with the highly significant differences in IL-10^+ Tr1% and $TGF\beta^+$ Th3%. While there have been reports of reduced $FOXP3^+$ Tregs in CF (217, 279), other studies have reported increases in regulatory cells in response to chronic inflammation (286, 295). This is consistent with our results, because we saw increases in regulatory subsets only in the CF adult population, nearly all of whom had experienced chronic infection/inflammation, with no increase seen in the CF children who had experienced very few respiratory infections.

It is also possible that the method of Treg identification used in this study may have contributed to the increased levels of FOXP3 Tregs that were observed. This study defined Tregs as $CD4^+FOXP3^+$ cells, but many other studies use additional markers such as CD25 and/or CD127 (199). Treg levels may also have appeared slightly higher because other more recently identified populations, such as $CD4^+$ T follicular regulatory and γδ T cells, have been

shown to have the ability to express FOXP3 (296, 297). This may also explain why the Treg% was significantly increased in CF but the Treg/Th17 ratio did not differ significantly between CF patients and controls.

This study has some weaknesses. First, Treg cells were analysed as a single population and we did not use additional markers such as CD45RA or CD45RO to identify naïve and memory Tregs, respectively, or any homing markers. Therefore, both of these additional analyses were performed in a subsequent set of experiments that are discussed in Chapter 4. Second, the only function of Treg cells that we analysed was their ability to produce their signature cytokines *in vitro* after polyclonal stimulation. It is therefore possible that while they may express the appropriate surface markers/cytokines, they may still differ functionally from those in healthy individuals. Indeed, it has been shown that inflammation-induced $FOXP3^+$ Tregs are functionally different from nTregs (286).

It was interesting to note an association of an increased $TGF\beta^+$ Th3% with a trend towards increased Th17% in patients with chronic *P. aeruginosa* infection but an absence of this association with any other common infection such as *S. aureus* or *A. fumigatus*. This may be because of the persistent and highly inflammatory nature of *P. aeruginosa* infection, but it also may be that, as previously suggested by Tiringer *et al*. (53), that the type of T cell subset analysis we performed could generate useful markers to monitor/predict *P. aeruginosa* infection. However, this cannot be determined from the current data.

One strength of this study is its relatively large numbers and broad age range compared with many similar studies. This allowed for a thorough analysis of the relationships between age groups and clinical variables with the T cell subsets measured, although a weakness is that some subgroups were too small to allow extended analyses of their differences. Other weaknesses include that this was a cross-sectional study and therefore none of the parameters measured provide any predictive ability or allow determination of causality. Additionally, lung function measurements were not available for every patient and not all of the measurements were performed on the same day as blood sampling.

In conclusion, this study shows for the first time that a high peripheral blood Th17% is associated with poorer lung function in people with CF and that this could potentially be used as an alternative marker of lung function. This could be particularly useful in young children (< 6 years) for whom standard lung function testing is not always possible. While several infant spirometry tests exist, they are generally invasive and are yet to be recognised as

essential for routine clinical care. Most CF children have annual routine blood tests for review of their condition, so the development of a blood test reflecting changes in lung function could prove to be very useful clinical tool.

The increase of TGFβ^{+} Th3% in relation to *P. aeruginosa* infection could also prove to be a useful clinical tool to predict changes in lung function. However, further studies are required to determine the prognostic value of this measurement in predicting the progression of CF-related lung disease.

CHAPTER 4

Flow Cytometric Analysis of Peripheral Blood $CD4^+$ T Cell Subset Homing Markers

4.1 Introduction

Trafficking of T cells to sites of infection and inflammation is vital for their ability to exert their functional capacity and produce an immune response. Chemokines are a family of cytokines with the ability to induce chemotaxis in cells with responsive receptors. Chemokines and their receptors are a major regulatory factor of, and are essential for, this migration of immune cells. A cascade of signalling events, relying heavily upon Ca^{2+} signalling, leads to the activation of chemokine receptors and the resulting migration of the cells that express them. Calcium signalling has been shown to be affected indirectly by defective expression of the CFTR chloride channel (276, 298), and Mueller and colleagues (22) have shown that this altered Ca^{2+} signalling results directly in aberrant cytokine signalling in mouse T cells. In addition, while there are no studies that have investigated this relationship in human T cells, a study by Assani *et al*. has shown that Ca^{2+} signalling is affected in human macrophages which affects their phagocytic function.

The most extensively studied chemokine in CF is the neutrophil-associated IL-8 (CXCL8), which acts via the CXCR1 and CXCR2 receptors and has been shown to be dysregulated in CF (241-243). Defects in not only chemokines but also chemokine receptors have also shown to have negative impacts in CF with reduced functioning of receptors such as CXCL9 leading to increased colonisation of pathogens such as *P. aeruginosa* (299). Many other studies of chemokines and their receptors in CF have focused on monocytes and macrophages, which also display varying levels of dysregulation (102, 244, 300). There is very little research involving chemokines and their receptors, specifically those associated with Th1-, Th2- and Th17-directed immune responses, in CF peripheral blood.

The presence in CF of altered ion signalling and neutrophil and macrophage/monocyte-associated chemokine signalling led us to hypothesise that this altered signalling would lead to alterations in chemokine receptor activation in T cells and thus T cell trafficking in people with CF, leading to ineffective immune responses. To investigate this, we stained PBMC from adult CF patients and healthy age-matched controls with antibodies for CD4, CD25, and CD127 to identify Treg cells, a more precise and newer identification technique for Tregs (199) than that described in Chapter 3, as well as for the chemokine receptors CXCR3, CCR4 and CCR6 to identify cells homing to sites of Th1-, Th2- and Th17-mediated inflammation, respectively. In addition to this the CD45RO marker was used to identify CD4 and Treg cells that were naïve ($CD45RO^-$) and memory ($CD45RO^+$) expressing these markers. The

participants with CF were further analysed to determine any correlations between subset proportions and relevant clinical variables.

4.2 Materials and Methods

4.2.1 PBMC Staining

Cryopreserved cells were used for all staining, and were resuscitated and prepared as described in Chapter 2. Specific antibody combinations used for staining for the experiments described in this chapter are listed in Table 9. Cells were counted using a Sysmex Cell Counter (Sysmex, NSW, Australia).

Table 9. Antibody combinations used to identify naïve and memory $CD4^+$ and Treg cells

Antibody	Marker	Fluorochrome	Hybridoma Clone	Supplier
Live/dead	Dead cells	Near infrared (IR)	-	ThermoFisher Scientific
anti-CD4	$CD4^+$ helper T cells	V500	RPA-T4	BD Biosciences
anti-CD25	Treg cells	Allophycocyanin (APC)	2A3	BD Biosciences
anti-CD127	Treg cells	Brilliant violet 421 (BV421)	A019D5	BioLegend
anti-CD45RO	Memory/naïve cells	BUV395	UCHL1	BD Biosciences
anti-CXCR3	Th1-associated cells	Alexa Fluor 488 (AF488)	1C6	BD Biosciences
anti-CCR4	Th2-associated cells	Phycoerythrin cyanin (PE-Cy7)	L291H4	BD Biosciences
anti-CCR6	Th17-associated cells	Phycoerythrin (PE)	11A9	BioLegend

4.2.2 Flow Cytometric Data Acquisition and Analysis

Stained cells were analysed using a 10-colour LSR II flow cytometer (BD Biosciences) with blue 488 nm, red 633 nm, violet 405 nm and UV 355 nm lasers to allow the measurement of 10 fluorescent and two scatter parameters using FACSDiva Software. Five hundred thousand events were collected per sample analysed. Data obtained from the flow cytometer was analysed using FlowJo software (Treestar). Figure 8 demonstrates the hierarchical gating strategy for identification of $CD4^+$ and Treg cells that express the CXCR3, CCR4 and CCR6 chemokine receptors.

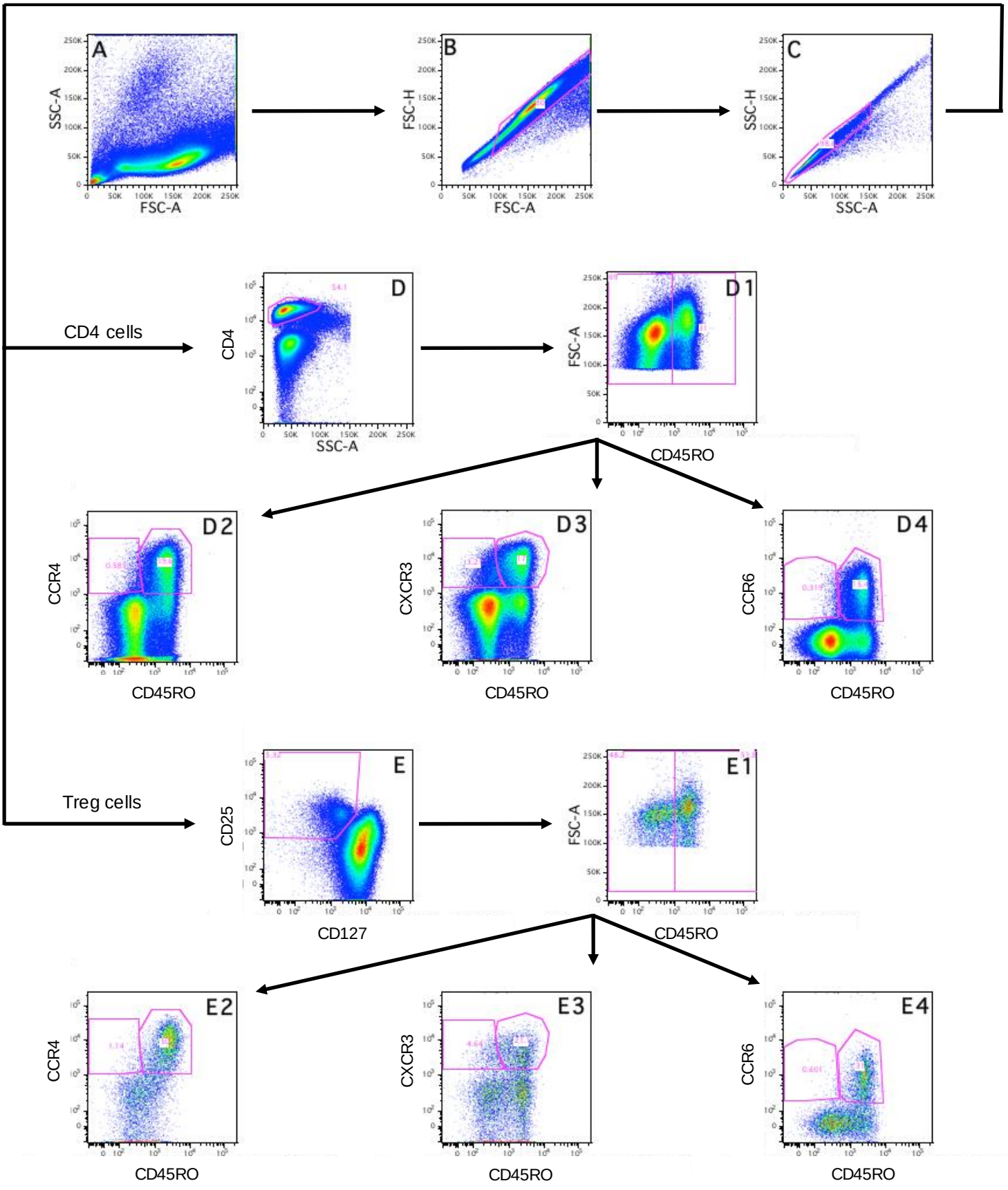

A
SSC-A
FSC-A
B
FSC-H
FSC-A
C
SSC-H
SSC-A
CD4 cells
D
CD4
SSC-A
D1
FSC-A
CD45RO
D2
CCR4
CD45RO
D3
CXCR3
CD45RO
D4
CCR6
CD45RO
Treg cells
E
CD25
CD127
E1
FSC-A
CD45RO
E2
CCR4
CD45RO
E3
CXCR3
CD45RO
E4
CCR6
CD45RO

Fig. 8. Representative histograms from healthy individual demonstrating the gating strategy for flow cytometry analysis of naïve and memory CD4+ and Treg cells expressing the chemokine receptors CCR4, CXCR3 and CCR6. 'A' shows ungated cells, 'B' and 'C' show gating of single cells, 'D' shows gating of $CD4^+$ cells, 'D1' shows gating of naïve and memory $CD4^+$ cells and 'D2', 'D3' and 'D4' show gating of naive and memory $CD4^+$ cells expressing CCR4, CXCR3 and CCR6 respectively. 'E' shows gating of Treg cells, 'E1' shows gating of naïve and memory Treg cells and 'E2', 'E3' and 'E4' show gating of Treg cells expressing CCR4, CXCR3 and CCR6, respectively.

4.3 Results

4.3.1 Comparison between CF and Control Groups

No significant differences were found between control and CF groups for median age, sex distribution, $CD4^+$%, Treg% or naïve/memory $CD4^+$% and Treg% (see Table 10), although there was a trend towards increased naïve Treg% and decreased memory Treg% in CF. It is important to note that here, Treg cells were identified as $CD4^+CD25^+CD127^{lo}$, in contrast to the analysis in Chapter 3 that identified them as $CD4^+FOXP3^+$, because since the experiments described in Chapter 3 were performed, it has become the standard technique to use these cell surface markers only and therefore avoid the cell permeabilisation required for FOXP3 staining (199). The percentages of memory $CD4^+$ cells expressing CXCR3 and CCR6 were significantly decreased in CF patients compared with controls, (P = 0.049 & 0.001 respectively) while there was no significant difference in the percentages of $CCR4^+$ memory $CD4^+$ cells. The percentages of memory Treg cells expressing CXCR3, CCR4 and CCR6 were all decreased in CF compared with controls (P = 0.025, 0.03 & 0.003 respectively). However, there were no significant differences between CF and controls in the percentages of any of the $CXCR3^+$, $CCR4^+$ and $CCR6^+$ naïve $CD4^+$ or Treg populations.

4.3.2 Comparison within the CF Cohort

We next performed an analysis of associations between the percentages of the measured cell populations and patient clinical parameters within the CF group, similar to that described in Chapter 2. Investigating those with (n = 3) or without (n = 15) current *A. fumigatus* infection and those with (n = 16) or without (n = 1) current treatment with any antimicrobial was not possible because of the small sizes of one of the groups. Tables 11, 12 and 13 detail all parameters assessed.

No significant differences were found in the percentages of any of the T cell subsets measured between different *CFTR* genotypes or between exacerbating and clinically stable patients.

FEV1 % predicted was first analysed in CF patients for whom spirometry and blood tests were performed within two weeks of each other (n = 13). A negative correlation was found between FEV1 % predicted and Treg% (r = –0.68, P = 0.010). FEV1 % predicted was then analysed in all the clinically stable patients (n = 14) and this relationship was still present (r = –0.72, P = 0.019). A trend was seen towards an inverse correlation of the percentages of $CCR4^+$ naïve (P = 0.06) and memory (P = 0.09) Treg cells and $CCR4^+$ naïve $CD4^+$ cells (P = 0.07) with FEV1 % predicted. Lastly, FEV1 % predicted was analysed only in stable patients

for whom spirometry and blood tests were performed within one week of each other (n = 5), because we had previously shown a time-dependent relationship between lung function and changes in peripheral blood T cell subset levels. Although findings from this analysis need to be considered cautiously given the small size of the group, we identified that percentages of both memory CCR4$^+$ CD4$^+$ (r = –1.00, P = 0.017) and Treg (r = –1.00, P = 0.017) cells were negatively correlated with FEV1 % predicted. As was the case for the experiments reported in Chapter 3, the relationship between total Treg% and FEV1 % predicted was no longer significant (P = 0.08).

When the CF group was divided based on the presence or absence of chronic *P. aeruginosa* infection, the naïve CXCR3 CD4$^+$% was found to be significantly lower (P = 0.014) in those with chronic *P. aeruginosa* infection, and there was a trend to lower memory CCR4$^+$CD4$^+$% (P = 0.06). No differences between the groups were seen for any of the Treg subsets measured.

Dividing the CF group based on *S. aureus* infection showed no differences in any of the CD4$^+$ subsets. The only difference noted in the regulatory subsets was a decrease in CXCR3$^+$Treg% in those with *S. aureus* infection (P = 0.0273).

Table 10. Comparison of percentages of $CCR4^+$, $CCR6^+$ and $CXCR3^+$ $CD4^+$ T cells and Treg cells in control and CF groups

	Control	CF	*P* value
N	15	17	
Age median (range)	28 (22–50)	25 (19–54)	0.50
Sex male (%)	5 (33%)	8 (47%)	
$CD4^+$ %* median (range)	34.00 (20.40–54.10)	34.60 (21.30–59.30)	0.94
$CD4^+RO^+$ %* median (range)	40.50 (19.90–58.40)	30.80 (15.00–61.00)	0.13
$CD4^+RO^-$ %* median (range)	59.50 (41.60–80.10)	69.20 (39.00–85.00)	0.13
$CD4^+CXCR3^+RO^+$ %* median (range)	18.40 (4.13–36.40)	13.20 (6.40–24.50)	**0.049**
$CD4^+CXCR3^+RO^-$ %* median (range)	2.54 (1.20–12.20)	2.53 (1.25–7.12)	0.94
$CD4^+CCR4^+RO^+$ %* median (range)	23.20 (7.90–38.60)	17.90 (9.69–38.30)	0.06
$CD4^+CCR4^+RO^-$ %* median (range)	1.53 (0.21–5.90)	1.22 (0.19–4.32)	0.46
$CD4^+CCR6^+RO^+$ %* median (range)	17.40 (7.43–34.70)	13.40 (2.94–19.10)	**0.001**
$CD4^+CCR6^+RO^-$ %* median (range)	0.33 (0.15–1.01)	0.30 (0.07–0.72)	0.26
Treg %* median (range)	7.33 (4.00–8.91)	7.85 (5.29–16.30)	0.39
Treg RO^+ %* median (range)	57.10 (40.30–77.60)	51.10 (30.40–91.30)	0.06
Treg RO^- %* median (range)	42.90 (22.40–59.70)	48.90 (8.72–69.60)	0.06
Treg $CXCR3^+RO^+$ %* median (range)	17.20 (2.81–30.10)	11.70 (3.30–21.20)	**0.025**
Treg $CXCR3^+RO^-$ %* median (range)	2.74 (1.00–8.14)	3.96 (0.71–7.22)	0.21
Treg $CCR4^+RO^+$ %* median (range)	50.00 (34.10–72.30)	44.90 (21.20–70.80)	**0.033**
Treg $CCR4^+RO^-$ %* median (range)	1.55 (0.53–7.08)	1.96 (0.46–8.76)	0.60
Treg $CCR6^+RO^+$ %* median (range)	33.70 (14.90–48.50)	21.70 (4.20–35.90)	**0.003**
Treg $CCR6^+RO^-$ %* median (range)	0.87 (0.33–2.67)	0.64 (0.12–3.20)	0.41
CD4+ $CD45RO^+$ CXCR3/CCR4 ratio median (range)	0.82 (0.18–1.51)	0.67 (0.15–1.59)	0.18
CD4+ $CD45RO^-$ CXCR3/CCR4 ratio median (range)	0.93 (0.39–29.26)	3.03 (0.31–19.35)	0.46
Treg $CD45RO^+$ CXCR3/CCR4 ratio median (range)	0.32 (0.05–0.62)	0.31 (0.07–0.53)	0.53
Treg $CD45RO^-$ CXCR3/CCR4 ratio median (range)	1.27 (0.32–7.40)	1.15 (0.23–8.67)	0.97
$CD45RO^-$ Treg/$CD4^+CCR6^+$ ratio median (range)	2.78 (1.54–8.13)	3.46 (2.17–12.48)	0.11
$CD45RO^+$ Treg/$CD4^+CCR6^+$ ratio median (range)	105.70 (42.48–311.3)	159.70 (55.19–382.9)	0.05

P values (Mann–Whitney *U* test) indicating significant differences are shown in **bold.**

* Indicates all values are calculated as a percentage of the parent population.

Table 11. Correlations between CCR4$^+$ CCR6$^+$ and CXCR3$^+$ naïve and memory CD4$^+$ T cell percentages and clinical variables in the CF group.

Parameter [no. of patients]	CD4$^+$ %*	CD4$^+$RO$^+$ %*	CD4$^+$RO$^-$ %*	CD4$^+$ CXCR3$^+$RO$^+$ %*	CD4$^+$ CXCR3$^+$RO$^-$ %*	CD4$^+$ CCR4$^+$RO$^+$ %*	CD4$^+$ CCR4$^+$RO$^-$ %*	CD4$^+$ CCR6$^+$RO$^+$ %*	CD4$^+$ CCR6$^+$RO$^-$ %*
Age [17] (Spearman *r*)	0.20	0.11	–0.11	-0.12	0.12	–0.04	0.01	–0.18	–0.14
***P* value**	[0.45]	[0.69]	[0.69]	[0.64]	[0.65]	[0.88]	[0.96]	[0.48]	[0.58]
FEV1 % pred. 2 weeks# all patients [13] (Spearman *r*)	–0.17	–0.14	0.14	0.35	0.34	–0.23	–0.12	0.17	0.29
***P* value**	[0.59]	[0.64]	[0.64]	[0.24]	[0.26]	[0.45]	[0.71]	[0.58]	[0.34]
FEV1 % pred. 2 weeks# stable patients only [10] (Spearman *r*)	–0.15	–0.14	0.14	0.39	0.26	–0.49	–0.60	0.06	0.42
***P* value**	[0.68]	[0.70]	[0.70]	[0.26]	[0.47]	[0.15]	[0.07]	[0.88]	[0.23]
FEV1 % pred. 1 week# stable patients only [5] (Spearman *r*)	0.10	–0.70	0.70	–0.10	0.60	–1.00	–0.70	–0.30	0.70
***P* value**	[0.95]	[0.23]	[0.23]	[0.95]	[0.35]	**[0.017]**	[0.23]	[0.68]	[0.23]
Median percent for each subset									
Sex									
Male	30.10	28.85	71.15	13.05	2.12	18.35	2.01	12.85	0.23
Female	40.30	33.10	66.90	15.40	2.73	16.60	0.52	13.90	0.33
***P* value**	[0.17]	[0.11]	[0.11]	[0.69]	[0.81]	[0.61]	[0.28]	[0.33]	[0.54]
Genotype									
DF508 [8]	37.50	32.80	67.20	14.15	2.36	17.70	0.87	13.00	0.30
DF508 heterozygous/other [9]	34.30	30.00	70.00	13.20	3.24	17.90	1.22	13.90	0.30
***P* value**	[0.54]	[0.42]	[0.42]	[0.59]	[0.61]	[0.89]	[0.81]	[0.56]	[0.74]
Stable [14]	36.20	30.40	69.60	13.05	2.43	18.35	1.28	12.25	0.27
Exacerbating [7]	31.60	33.10	66.90	15.60	2.53	19.00	2.20	13.80	0.33
***P* value**	[0.80]	[0.43]	[0.43]	[0.35]	[0.86]	[0.86]	[0.86]	[0.22]	[0.26]
Chronic *P. aeruginosa* [10]	34.45	34.30	65.70	13.20	1.94	19.75	1.28	13.30	0.302
No chronic *P. aeruginosa* [7]	43.50	30.50	69.50	15.40	3.76	13.30	0.59	13.90	0.303
***P* value**	[0.42]	[0.27]	[0.27]	[0.99]	**[0.014]**	[0.06]	[0.48]	[0.46]	[0.81]
Current *S. aureus* infection [11]	34.30	30.50	69.50	12.50	2.53	17.30	1.52	12.60	0.30
No *S. aureus* infection [6]	35.80	34.30	65.70	16.90	2.46	18.65	0.82	14.55	0.30
***P* value**	[0.99]	[0.22]	[0.22]	[0.10]	[0.88]	[0.35]	[0.66]	[0.13]	[0.88]

[*P* values] (Mann–Whitney *U* test) indicating significant differences are shown in **bold.**

*****Indicates values are calculated as a percentage of the parent population, # Indicates number of weeks between blood and spirometry testing.

Table 12. Correlations between CCR4+ CCR6+ and CXCR3+ naïve and memory Treg cell percentages and clinical variables in the CF group

Parameter [no. of patients]	Treg %*	Treg RO^+ %*	Treg RO^- %*	Treg $CXCR3^+RO^+$ %*	Treg $CXCR3^+RO^-$ %*	Treg $CCR4^+RO^+$ %*	Treg $CCR4^+RO^-$ %*	Treg $CCR6^+RO^+$ %*	Treg $CCR6^+RO^-$ %*
Age [17] (Spearman *r*)	0.17	0.12	–0.12	–0.18	–0.08	0.03	0.06	–0.02	–0.07
***P* value**	[0.52]	[0.66]	[0.66]	[0.48]	[0.76]	[0.90]	[0.82]	[0.94]	[0.79]
FEV1% pred. 2 weeks# all patients [13] (Spearman *r*)	–0.68	–0.16	0.16	0.36	0.28	–0.17	–0.19	–0.09	0.07
***P* value**	**[0.010]**	[0.60]	[0.60]	[0.22]	[0.36]	[0.58]	[0.54]	[0.78]	[0.83]
FEV1% pred. 2 weeks# stable patients only [10] (Spearman *r*)	–0.72	–0.47	0.47	0.39	0.52	–0.55	–0.61	–0.25	0.39
***P* value**	**[0.019]**	[0.17]	[0.17]	[0.26]	[0.13]	[0.09]	[0.06]	[0.49]	[0.26]
FEV1% pred. 1 week# stable patients only [5] (Spearman *r*)	–0.90	–0.90	0.90	0.10	0.80	–1.00	–0.60	–0.30	0.70
***P* value**	[0.08]	[0.08]	[0.08]	[0.95]	[0.13]	**[0.017]**	[0.35]	[0.68]	[0.23]
Median percent for each subset									
Sex									
Male	8.20	52.05	47.95	13.00	3.49	45.45	3.35	23.65	0.89
Female	7.24	48.10	51.90	11.10	3.96	40.20	1.72	16.90	0.55
***P* value**	[0.09]	[0.48]	[0.48]	[0.42]	[0.74]	[0.28]	[0.24]	[0.09]	[0.74]
Genotype									
DF508 homozygous [8]	7.80	49.60	50.40	9.75	3.64	42.55	1.94	16.60	0.53
DF508 heterozygous/other [9]	8.05	51.20	48.80	12.00	4.21	46.00	2.05	23.00	0.78
***P* value**	[0.27]	[0.54]	[0.54]	[0.61]	[0.89]	[0.37]	[0.67]	[0.11]	[0.48]
Stable [14]	7.86	49.65	50.35	11.85	3.69	44.65	2.01	20.60	0.49
Exacerbating [7]	7.85	51.10	48.90	11.20	3.96	45.10	4.04	16.90	0.64
***P* value**	[0.68]	[0.54]	[0.54]	[0.99]	[0.50]	[0.50]	[0.80]	[0.74]	[0.22]
Chronic *P. aeruginosa* [10]	8.12	52.00	48.00	11.60	3.27	45.00	2.01	20.60	0.612
No chronic *P. aeruginosa* [7]	7.85	48.10	51.90	11.70	4.79	40.20	1.72	23.00	0.640
***P* value**	[0.58]	[0.36]	[0.36]	[0.99]	[0.32]	[0.32]	[0.54]	[0.96]	[0.67]
Current *S. aureus* infection [11]	7.85	43.50	56.50	10.40	3.96	33.10	3.04	18.80	0.67
No *S. aureus* infection [6]	7.24	50.90	49.10	16.05	4.06	44.75	1.94	24.05	0.53
***P* value**	[0.87]	[0.18]	[0.18]	**[0.027]**	[0.99]	[0.30]	[0.66]	[0.15]	[0.46]

[*P* values] (Mann–Whitney *U* test) indicating significant differences are shown in bold.

*****Indicates values are calculated as a percentage of the parent population. #Indicates number of weeks between blood and spirometry testing.

4.3.3 CXCR3/CCR4 (Th1/Th2) Ratios within the CF Group

Because of the many reports of a Th2 bias in CF, we compared the ratios of $CXCR3^+/CCR4^+$ $CD4^+$ and Treg cells between the CF and control groups (Table 13), but found no significant differences.

The CXCR3/CCR4 ratios were also analysed within the CF cohort using the same parameters used for the other subsets (Table 13). No significant associations were found between the ratios of $CXCR3^+/CCR4^+$ $CD4^+$ and Treg cells and sex, genotype, stable/exacerbating clinical status or the presence/absence of *S. aureus* infection.

When CF patients were divided based on the presence of chronic *P. aeruginosa* infection, those patients with chronic *P. aeruginosa* infection had lower naïve $CD4^+$ $CXCR3^+/CCR4^+$ ratios compared with patients without chronic *P. aeruginosa* infection ($P = 0.025$), indicating that they had lower levels of Th1-associated and higher levels of Th2-associated naïve $CD4^+$ cells.

When analysing the $CXCR3^+/CCR4^+$ ratios in relation to lung function for patients for whom spirometry was available within two weeks of blood testing (n = 13), no significant associations were found, although there was a trend towards an increase in FEV1 % predicted with increased memory $CD4^+CXCR^+/CCR4^+$ ratio ($P = 0.09$). When only the clinically stable patients were included in this analysis (n = 14), a positive correlation was found between chronic *P. aeruginosa* infection and the memory $CD4^+$ $CXCR3^+/CCR4^+$ ratio (r = 0.75, $P = 0.013$), while there was a trend towards a positive correlation between FEV1 % predicted and the naïve $CD4^+$ $CXCR3^+/CCR4^+$ ratio ($P = 0.09$). Naïve Treg $CXCR3^+/CCR4^+$ cells were positively correlated with FEV1 % predicted (r = 0.64, $P = 0.048$) while the memory Treg $CXCR3^+/CCR4^+$ ratio trended towards being positively correlated with FEV1 % predicted ($P = 0.054$). Analysis of clinically stable patients for whom blood and spirometry tests were performed within one week of each other (n = 5) showed no significant differences in $CXCR3^+/CCR4^+$ ratios; however, there was a trend towards a positive correlation between FEV1 % predicted and both naïve and memory Treg and memory $CD4^+$ $CXCR3^+/CCR4^+$ ratios (all P values = 0.083).

4.3.4 Treg/$CD4^+CCR6^+$ (Treg/Th17) Ratios within the CF Group

As Treg/Th17 imbalances are often reported to cause immune pathology, the Treg/$CD4^+CCR6^+$ ratio was investigated but there was no significant difference between the CF

and control groups (Table 13). The Treg/$CD4^+CCR6^+$ ratio was also analysed within the CF cohort using the same parameters used for the other subsets (Table 13). However, no significant associations were found between the Treg/$CD4^+CCR6^+$ ratio and any of the clinical variables.

Table 13. Correlations between ratios of naïve and memory T cells and clinical variables in the CF group

Parameter [no. of patients]	$CD4^+RO^+$ CXCR3/CCR4 ratio	$CD4^+RO^-$ CXCR3/CCR4 ratio	Treg RO^+ CXCR3/CCR4 ratio	Treg RO^- CXCR3/CCR4 ratio	RO^+ Treg/$CD4^+CCR6^+$ ratio	RO^- Treg/$CD4^+CCR6^+$ ratio
Age [17] (Spearman *r*)	–0.25	0.05	–0.26	–0.13	0.16	0.13
***P* value**	[0.34]	[0.84]	[0.31]	[0.62]	[0.54]	[0.62]
FEV 1% pred. 2 weeks# all patients [13] (Spearman *r*)	0.49	0.26	0.47	0.28	0.00	–0.07
***P* value**	[0.09]	[0.39]	[0.12]	[0.35]	[1.00]	[0.83]
FEV 1% pred. 2 weeks# stable patients only [10] (Spearman *r*)	0.75	0.55	0.62	0.64	–0.01	–0.27
***P* value**	**[0.013]**	[0.09]	[0.054]	**[0.048]**	[0.99]	[0.45]
FEV 1% pred. 1 week# stable patients only [5] (Spearman *r*)	0.90	0.60	0.90	0.90	–0.10	0.10
***P* value**	[0.08]	[0.35]	[0.08]	[0.08]	[0.95]	[0.95]
Median percent for each subset						
Sex						
Male	0.79	1.31	0.27	0.88	147.7	3.99
Female	1.00	6.66	0.35	2.75	198.3	3.24
***P* value**	[0.20]	[0.20]	[0.42]	[0.32]	0.37	0.42
Genotype						
DF508 homozygous [8]	0.82	4.15	0.33	1.88	187.2	3.35
DF508 heterozygous/other [9]	0.84	3.03	0.28	1.15	155.5	3.92
***P* value**	[0.96]	[0.82]	[>0.99]	[0.89]	0.28	0.67
Stable [14]	0.70	2.34	0.24	1.04	138.9	3.41
Exacerbating [7]	0.84	1.51	0.32	1.02	147.7	3.16
***P* value**	[0.23]	[0.97]	[0.69]	[0.80]	0.74	0.44
Chronic *P. aeruginosa* [10]	0.75	1.45	0.28	0.98	151.6	3.30
No chronic *P. aeruginosa* [7]	0.91	22.12	0.31	3.30	170.0	3.92
***P* value**	[0.27]	**[0.025]**	[0.42]	[0.16]	0.81	0.54
Current *S. aureus* infection [11]	0.57	3.03	0.28	1.15	162.8	3.64
No *S. aureus* infection [6]	0.84	4.15	0.38	1.78	130.7	3.35
***P* value**	[0.40]	[0.66]	[0.30]	[0.99]	0.43	0.96

[*P* values] (Mann–Whitney *U* test) indicating significant differences are shown in **bold.**

# Indicates number of weeks between blood and spirometry testing.

4.4 Discussion

Balanced T helper responses are essential to prevent immunopathology resulting from an immune response to pathogens. Studies have shown that a Th2-mediated immune response dominates in CF patients with *P. aeruginosa* (247, 262, 301) and *A. fumigatus* (52, 302-304) infections, and that this bias impairs efficient clearance of these infections. Our study, which investigated chemokine receptors on both memory and naïve $CD4^+$ and Treg cells in the peripheral blood of CF patients, demonstrates for the first time that CCR4 (Th2-associated) expression on peripheral $CD4^+$ effector and regulatory T cell subsets and the ratio of $CXCR3^+/CCR4^+$ memory $CD4^+$ cells are correlated with FEV1 % predicted in clinically stable CF patients. Because the patients' clinical condition can change quite rapidly and because our previous findings showed that the relationship between peripheral blood Th17% and lung function was very time-dependent, no data involving measurements of lung function beyond a two-week period around the blood sampling for CD4 subset analysis were included. The relationship shown between the $CXCR3^+/CCR4^+$ ratio and FEV1 % predicted indicates that a Th1-mediated immune response to lung infection may be more beneficial for maintaining lung function. Supporting this concept is the negative correlation observed between FEV1 % predicted and the percentages of Th2-associated $CCR4^+CD4^+$ cells and $CCR4^+$Treg cells within the same group of clinically stable patients. We were unable to access samples to measure T cell subsets in the patients' lungs, but it is possible that the high levels of Th2-associated and low levels of Th1-associated cells in peripheral blood of patients with poor lung function could reflect the levels of these cells in the lungs. The correlation between higher Th2-associated cells and poorer lung function would be consistent with the results of previous studies showing that a Th2-type response is less effective than a Th1-type response (249) in combating CF-related pathogens.

Alterations in the $CXCR3^+/CCR4^+$ ratio were found not only for total $CD4^+$ cells but also for Treg cells. A positive association was found between naïve Treg cells and FEV1 % predicted ($P = 0.048$) as well as a trend towards the same association between memory Treg cells and FEV1 % predicted ($P = 0.054$). This could be explained by the fact that Th1-associated regulatory cells are required to suppress the Th1 cell-mediated inflammation that can be damaging to tissues during prolonged inflammation (305).

It is important to note that in CF peripheral blood, the proportions of both memory $CD4^+$ and memory Treg cells expressing CXCR3 and CCR6 were decreased, while the proportion of

$CCR4^+$ cells was decreased only for memory Treg cells not memory $CD4^+$ cells. Therefore, the overall levels of $CCR4^+$ regulatory cells were decreased while those of $CCR4^+$ effector cells were unchanged. This apparent imbalance of effector and regulatory $CCR4^+$ cells suggests that there are fewer Th2-associated regulatory cells to suppress Th2-associated effector cells, which could potentially contribute to the Th2 bias observed in CF. This imbalance could potentially be a target of therapy to reduce overactive Th2 immune responses and improve clearance of respiratory pathogens.

Lastly, decreased percentages of naïve $CXCR3^+CD4^+$ cells were seen in patients with chronic *P. aeruginosa* infection. This could be because $CXCR3^+$ Th1 $CD4^+$ cells, which are known to be more effective than Th2 cells at clearing *P. aeruginosa,* have homed to the lung-draining lymph nodes in response to the presentation of *P. aeruginosa* antigens by APC there. However, an alternative explanation suggested by Lazarski and colleague's study (306), which showed that IL-4, a signature Th2 cytokine, limits trafficking of Th1 cells to inflamed tissues, is that because of the Th2 dominance observed in the lungs of CF patients with chronic *P. aeruginosa* infection, the Th1-associated cells may not make it into the lungs to produce an effective immune response. Yet another potential explanation is that long-term Th2 dominance may have an effect on Th1 cell development at the gene expression level. Expression of Th2-associated genes is known to suppress Th1 cell development through inhibition of key Th1-regulating transcription factors (307). Prolonged inhibition of expression of these genes could therefore potentially affect the generation of new Th1 cells. However, given that the memory $CXCR3^+CD4^+$% appears unaffected in patients with *P. aeruginosa* infection, this may not be important in that context. The reduction of naïve $CXCR3^+CD4^+$% also has an effect on the $CXCR3^+/CCR4^+$ ratio for naïve $CD4^+$ cells, but because this ratio showed no relationship with any relevant clinical parameters including lung function, this could therefore be considered inconsequential. However, given that low percentages of naïve $CXCR3^+CD4^+$ cells are specific to patients with *P. aeruginosa,* it is also possible that this could be a marker for monitoring/predicting *P. aeruginosa* infection, although this cannot be determined from the current data.

The only previous study in CF patients that has assessed CXCR3 and CCR4 expression (247) evaluated these chemokine receptors on $CD4^+$ and $CD8^+$ T cells in BALF. The primary findings from that study all related to *P. aeruginosa* infection, and reported elevated levels of $CCR4^+CD4^+$ cells and of the Th2-related cytokines IL-4 and IL-13, as well as an association between these cytokines and *P. aeruginosa* infection and poorer lung function. Consistent

with this, Tiringer and colleagues (53) showed that high levels of Th2 and Th17 cells preceded *P. aeruginosa* infection. The correlations observed between the peripheral memory $CD4^+CXCR3^+/CCR4^+$ ratio and memory $CCR4^+CD4^+$% and Treg% and the FEV1 % predicted in clinically stable CF patients may therefore be a useful indicator of susceptibility to *P. aeruginosa* acquisition and therefore lung function decline. However, because we observed that the association between these T cell subset percentages and FEV1 % predicted was only detected within a tight time frame, additional research is required to determine whether there is any prognostic value for lung function decline in measuring Th1/Th2 related markers in peripheral blood.

Studies in non-CF patients infected with pathogens such as *Mycobacterium tuberculosis*, *A. fumigatus* and *Leishmania spp* (302, 306, 308) have all demonstrated the presence of a Th2-biased response to infection. A study of patients with idiopathic pulmonary fibrosis has also implicated imbalances of CXCR3 and CCR4 expression on BALF cells in disease progression (309). These studies suggest that the alterations in Th1/Th2 associated markers observed in our study may not be specific to CF. It has been suggested that a high microbial burden in infected patients can lead to suppression of the cell-mediated Th1 response and dominance of the humoral-mediated Th2 response as a mechanism to prevent excessively damaging cell-mediated immune responses that can potentially cause more harm than the pathogen (158, 305). In addition, the generation of Th2 cells producing cytokines such as IL-4, IL-5 and IL-13 produces a cytokine milieu conducive to the further generation of Th2 cells and inhibition of Th1 cells (305), and Lazarski *et al.* (306) showed that IL-4, a signature Th2 cytokine, not only promotes further Th2 cell generation but also limits trafficking of Th1 cells to inflamed tissues. Therefore, all of these could be contributing factors to altered peripheral T cell subset levels in CF.

It is notable that although many differences between CF patients and healthy controls were observed among the memory $CD4^+$ and Treg cell populations expressing CXCR3 (Th1-associated), CCR4 (Th2-associated) and CCR6 (Th17-associated), no differences were found for any naïve subset. This may indicate that although in CF patients there are normal levels of 'inexperienced' cells able to differentiate into effector and regulatory cells upon antigen stimulation, either their ability to differentiate to $CXCR3^+$ and $CCR6^+$ memory cells is impaired or the memory cells have homed to sites away from the periphery. It would appear most logical that the decrease in these cells is because of their homing to the lungs, where there is a high concentration of proinflammatory cytokines and chemokines to combat the

chronic infection in the lungs. The reported overactive Th2 response in the lungs may also be reflected in the periphery and could explain why, in contrast to those of $CXCR3^+$ and $CCR6^+$ memory $CD4^+$ cells, the percentage of $CCR4^+$ memory T cells is not decreased in the periphery. This is consistent with a study by Häusler and colleagues (140) that investigated memory $CD4^+$ T cells as a whole population, rather than identifying specific subsets, and showed a decrease of $CD4^+$ memory cells in CF peripheral blood but an increase of these cells in CF lung tissue, suggesting that it is possible that memory $CD4^+$ T cells home from the periphery to the lungs in CF.

It is interesting to note that the percentages of Tregs defined using CD4, CD25 and CD127 rather than the CD4 and FOXP3 markers used in the previous experiments described in Chapter 3, are still negatively correlated with FEV1 % predicted. Although two previous studies in CF have shown peripheral blood Tregs to be positively correlated with FEV1 % predicted (217, 279), those studies included mainly infants and adolescents who were yet to experience prolonged infection, in contrast with our cohort who were all adults (>18 years), many with chronic infections. Because it has been reported that Tregs undergo an age- and disease progression-dependent impairment of their suppressive ability (217), it is possible that in older patients, increasing numbers of Tregs are induced because they become less effective.

The main strength of this study is the detailed analysis that combines both chemokine receptor markers and a naïve/memory marker for both $CD4^+$ effector and regulatory T cells. One weakness is that this is a cross-sectional study and therefore none of the parameters measured provide any predictive ability or allow determination of causality. A second weakness is that the levels of these cells were not measured in the lung or BALF (site of infection) or in the lymph nodes (site of antigen presentation) to provide a true picture of where the T cells are homing in response to infection.

In conclusion, we have shown that the level of $CD4^+$ T cells expressing CXCR3 and CCR4 chemokine receptor in peripheral blood is reflective of lung function in clinically stable CF patients. These markers, like peripheral blood Th17%, could potentially be used as relatively noninvasive markers of lung function. The decrease in naïve $CXCR3^+CD4^+$% in relation to *P. aeruginosa* infection could also have potential as a clinical tool to predict changes in lung function.

Peripheral percentages of $CXCR3^+$, $CCR4^+$ and $CCR6^+$ memory Treg cells were decreased in CF compared with healthy controls while only $CXCR3^+$ and $CCR6^+$ memory CD4 cells were

decreased, resulting in an imbalance of Th2-associated effector and regulatory cells. Correction of this imbalance to dampen overactive Th2 immune responses could potentially improve clearance of respiratory pathogens in CF patients.

CHAPTER 5

Flow Cytometric Analysis of Peripheral Blood Monocytes, Natural Killer (NK) cells, Dendritic Cells (DC) and Myeloid Derived Suppressor Cells (MDSC)

5.1 Introduction

Effective communication between the innate and adaptive immune system is essential for mounting appropriate immune responses. Destruction of lung tissue, in which the immune system is thought to play a major role, is the primary cause of morbidity and mortality in CF. In recent years, innate immune cells and their subsequent effects on adaptive immune responses have become an area of interest in understanding immune dysregulation in CF and its effects on lung damage. While we (310) and others (20, 220, 262, 290) have shown dysregulation of adaptive $CD4^+$ T cells in CF, there is far less research investigating how innate immune cells other than neutrophils are contributing to this dysregulation. In particular, much of the research involving the innate immune response is based primarily at the site of infection, in the lungs.

Cell types such as monocytes/macrophages (311, 312) and DCs (23) are of particular interest because they are also known to normally express CFTR and they directly influence T cells through antigen presentation. Other cell types that have not been confirmed to express CFTR, such as NK cells and MDSCs, are also of interest because of their prominent role in controlling infection and inflammation. Therefore, investigating changes in these cells away from the lungs and the direct influence of infection and inflammation may provide insight about intrinsic defects in these cell types.

To investigate innate immune cells in CF, we stained PBMC from adult CF patients and healthy age-matched controls with antibodies to identify monocytes, NK cells, DCs and MDSCs and analysed these populations using flow cytometry. The CF group data were also analysed to determine any correlations between subset proportions and relevant clinical variables.

5.2 Materials and Methods

5.2.1 PBMC Staining

Cryopreserved cells were used for all staining, and were resuscitated and prepared as described in Chapter 2. The specific antibody combinations used for staining for the experiments described in this chapter are listed in Table 14. Cells were counted using a Sysmex Cell Counter (Sysmex, NSW, Australia).

Table 14. Antibody combinations used for identification of monocytes, natural killer, dendritic and myeloid derived suppressor cells

Antibody	Marker	Fluorochrome	Hybridoma Clone	Supplier
Live dead	Dead cells	Near infrared (IR)	–	ThermoFisher Scientific
anti-CD3	T cells	Biotin	OKT3	Centenary Institute, Newtown, NSW, Aus
anti-avidin	Biotin labelled cells (T and B cells)	Pacific orange (PO)	–	Centenary Institute
anti-CD19	B cells	Biotin	HIB19	BD Biosciences
anti-CD14	Monocytes Myeloid derived suppressor cells	Pacific Blue (PB)	M5E2	BD Biosciences
anti-CX3CR1	Monocytes Dendritic Cells	Fluorescein isothiocyanate (FITC)	2A9-1	BioLegend
anti-NKP46	Natural killer cells	Phycoerythrin (PE)	9E2	BD Biosciences
anti-CD16	Natural killer cells Monocytes Myeloid derived suppressor cells	Peridinin chlorophyll protein cyanin 5.5 (PerCP-Cy5.5)	3G8	BD Biosciences
anti-CD11b	Myeloid derived suppressor cells	Phycoerythrin cyanin (PE-Cy7)	ICRF44	BD Biosciences
anti-CD56	Natural killer cells	Allophycocyanin (APC)	NCAM16.2	BD Biosciences

5.2.2 Flow Cytometric Data Acquisition and Analysis

Stained PBMCs were analysed using a BD FACSCanto II nine-colour flow cytometer (BD Biosciences) with blue 488 nm, red 633 nm and violet 405 nm lasers allowing measurement of nine fluorescence and two scatter parameters using FACSDiva software (BD Biosciences). Five hundred thousand events were collected per sample analysed. Data obtained from the flow cytometer were analysed using FlowJo software (Treestar, Ashland, OR). Figure 9 demonstrates the hierarchical gating strategy for identification of cells expressing markers of monocytes, NK cells, DCs and MDSC. Monocyte, MDSC, NK and DC populations were analysed as percentages of 'non T and B cells' or as percentages of their parent population where appropriate. Measurement of these cells as a percent of 'non-T non-B cells' allows identification of the proportions of a particular cell type within the entire population of cells of interest while measurement of cells as a percent of the parent population allows identification of the changes in proportions of a subpopulation within a given cell type.

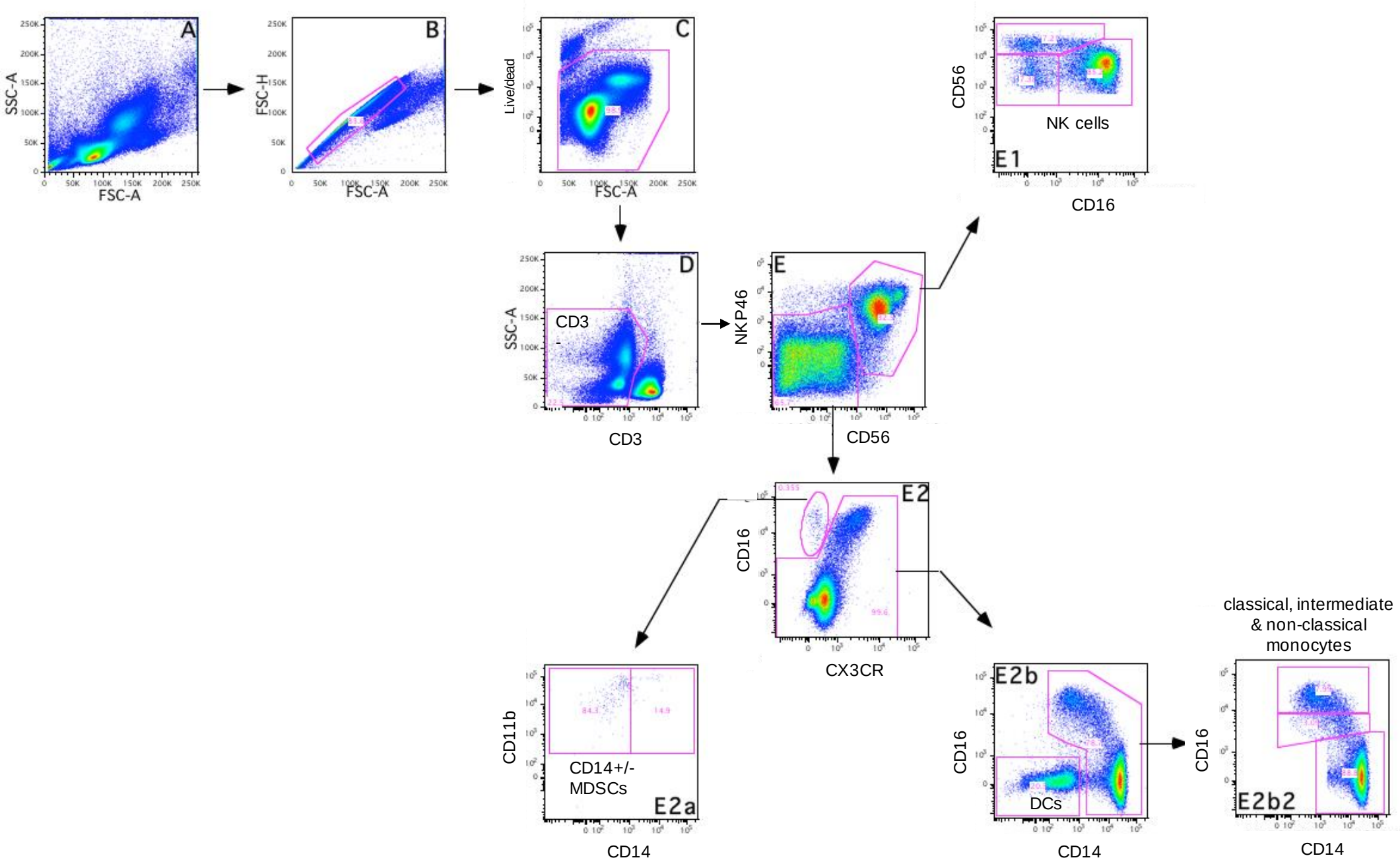

A
SSC-A
FSC-A
B
FSC-H
FSC-A
C
Live/dead
FSC-A
D
SSC-A
CD3
-
CD3
E
NKP46
CD56
E1
CD56
NK cells
CD16
E2
CD16
CX3CR
E2a
CD11b
CD14+/-
MDSCs
CD14
E2b
CD16
DCs
CD14
E2b2
CD16
CD14
classical, intermediate
& non-classical
monocytes

Fig. 9. Representative histograms from healthy individual demonstrating the gating strategy for flow cytometry analysis of monocytes, natural killer cells, dendritic cells and myeloid-derived suppressor cells. 'A' shows ungated cells, 'B' shows gating of single cells, 'C' shows gating of live cells and 'D' shows gating of non-T and B cells. 'E' shows gating on a population containing natural killer cells and a population containing monocytes, dendritic cells and myeloid-derived suppressor cells. 'E1'shows gating of natural killer cell subpopulations. 'E2' shows gating of myeloid-derived suppressor cells and a population containing monocytes and dendritic cells. 'E2a' shows gating of myeloid-derived suppressor cell subpopulations. 'E2b' shows gating of dendritic cells and monocytes and 'E2b2' shows gating of monocyte subpopulations.

5.3 Results

5.3.1 Comparison between CF and Control Groups

There was no significant difference in the median age or the sex distribution of the control and CF groups. A comparison of the flow cytometry results for the two groups is outlined in Table 15. Overall percentages of MDSCs, measured as a percent of non-T non-B cells, did not differ significantly between the control and CF groups, so we next focussed on analysis of subpopulations. First, MDSCs were divided based on CD14 expression to identify monocytic ($CD14^{+}$) and granulocytic ($CD14^{-}$) populations. When measured as a percent of the parent (MDSC) population, $CD14^{+}$ MDSCs were increased in CF patients compared with controls (P = 0.0115) while $CD14^{-}$ MDSCs were decreased in CF (P = 0.004). However, neither population differed significantly between CF and control groups when measured as a percent of non-T non-B cells. DCs, which were analysed as a single population, were increased in CF patients compared with controls (P = 0.0158).

The overall monocyte population was significantly increased in CF compared with controls (P = 0.0214). Three subpopulations of monocytes were identified based on CD14 and CD16 expression: $CD14^{hi}CD16^{lo}$, $CD14^{int}CD16^{int}$ and $CD14^{lo/neg}CD16^{hi}$, and the levels of these were calculated as a percent of the parent (monocyte) population as well as a percent of 'non-T non-B' cells. $CD14^{hi}CD16^{lo}$ 'classical' monocytes were increased in CF compared with controls when defined as either a percent of the parent population (P = 0.0331) or as a percent of 'non-T non-B' cells (P = 0.002). $CD14^{int}CD16^{int}$ 'intermediate' monocytes were unchanged in CF compared with controls. $CD14^{lo/neg}CD16^{hi}$ 'non-classical' monocytes were decreased when measured as a percent of 'non-T non-B' cells (P = 0.0412) but were unchanged when measured as a percent of the parent population.

The overall NK cell population, identified by NKP46 expression, was decreased in CF compared with controls (P = < 0.0001). When NK cells were further divided into subpopulations based on CD16 and CD56 expression, three distinct populations were identified, $CD16^{hi}CD56^{int}$, $CD16^{lo/neg}CD56^{hi}$ and $CD16^{lo/neg}CD56^{int}$, as described in section 1.2.2.5. All of these subsets were measured as a percent of the parent population (NK cells) and of 'non-T non-B' cells. The $CD16^{hi}CD56^{int}$ cytotoxic NK cells were significantly decreased in CF compared with controls when calculated as either a percent of parent population (P = <0.0001) or as a percent of 'non-T non-B' cells (P = < 0.0001). The $CD16^{lo/neg}CD56^{hi}$ cytokine-producing subpopulation was significantly increased in CF

compared with controls (P = < 0.0001) when calculated as a percent of the parent population, but did not differ significantly between the groups when calculated as a percent of 'non-T non-B' cells. $CD16^{lo/neg}CD56^{int}$ cytotoxic NK cells showed a trend towards an increase in CF compared with controls (P = 0.0695) when calculated as a percent of the parent population, but did not differ significantly when they were calculated as a percent of 'non-T non-B' cells.

Table 15. Comparison of monocyte, natural killer, myeloid derived suppressor and dendritic cell subset percentages between control and CF groups

	Control	CF	*P* value
N	15	17	
Age median (range)	28 (22–50)	25 (19–54)	0.4947
Sex male (%)	5 (33%)	8 (47%)	
MDSC (% non-T non-B cells) median (range)	0.44 (0.15–1.72)	0.36 (0.03–1.33)	0.1922
$CD14^+$ MDSC (% non-T non-B cells) median (range)	0.035 (0.006–0.346)	0.047 (0.001–0.389)	0.3310
$CD14^+$ MDSC (% MDSC) median (range)	8.27 (1.06–25.20)	15.10 (5.56–34.60)	**0.0115**
$CD14^-$ MDSC (% non-T non-B cells) median (range)	0.40 (0.14–1.52)	0.23 (0.02–0.99)	0.1206
$CD14^-$ MDSC (MDSCs) median (range)	91.00 (74.80–98.80)	83.30 (65.40–92.60)	**0.0040**
Dendritic Cells (% non-T non-B cells) median (range)	12.50 (8.66–22.30)	18.90 (6.07–49.80)	**0.0158**
Monocytes (% non-T and B cells) median (range)	34.90 (20.00–56.10)	46.00 (24.60–76.70)	**0.0214**
$CD16^{hi}CD14^{lo}$ Monocytes (% non-T non-B cells) median (range)	1.68 (0.49–4.80)	4.50 (0.65–8.37)	**0.0020**
$CD16^{hi}CD14^{lo}$ Monocytes (% monocytes) median (range)	6.05 (1.24–15.70)	7.65 (1.93–22.90)	**0.0331**
$CD16^{int}CD14^{int}$ Monocytes (% non-T non-B cells) median (range)	1.27 (0.51–2.68)	3.04 (1.47–7.04)	0.1203
$CD16^{int}CD14^{int}$ Monocytes (% monocytes) median (range)	4.40 (1.53–6.25)	1.55 (0.59–4.24)	0.4497
$CD16^{lo/neg}CD14^{hi}$ Monocytes (% non-T non-B cells) median (range)	88.80 (78.40–97.20)	37.20 (20.40–65.00)	**0.0412**
$CD16^{lo/neg}CD14^{hi}$ Monocytes (% monocytes) median (range)	63.10 (40.20–76.90)	88.70 (70.20–92.70)	0.3647
Natural Killer Cells (% non-T non-B cells) median (range)	49.50 (26.50–66.50)	23.10 (9.31–41.40)	**<0.0001**
$CD16^{hi}CD56^{int}$ NK (% non-T non-B cells) median (range)	42.60 (21.70–60.80)	18.20 (1.43–33.70)	**<0.0001**
$CD16^{hi}CD56^{int}$ NK (% NK cells) median (range)	89.10 (80.20–94.90)	76.70 (15.30–91.00)	**<0.0001**
$CD16^{lo/neg}CD56^{hi}$ NK (% non-T non-B cells) median (range)	3.03 (1.41–3.95)	3.29 (0.68–9.27)	0.3954
$CD16^{lo/neg}CD56^{hi}$ NK (% NK cells) median (range)	5.94 (2.35–11.30)	16.70 (3.01–50.20)	**<0.0001**
$CD16^{lo/neg}CD56^{int}$ NK (% non-T non-B cells) median (range)	2.07 (0.99–7.44)	1.84 (0.38–4.48)	0.3699
$CD16^{lo/neg}CD56^{int}$ NK (% NK cells) median (range)	5.17 (1.90–15.00)	6.64 (3.59–48.10)	0.0695

P values indicating significant differences are shown in **bold.**

5.3.2 Comparisons within the CF Cohort

We next investigated any important associations between the measured cell populations and clinical parameters within the CF group, as described in Chapter 2. Investigating those with (n = 3) or without (n = 15) current *A. fumigatus* infection and those with (n = 16) or without (n = 1) current treatment with any antimicrobial was not possible given the small sizes of one of the groups. Tables 16, 17 and 18 detail all parameters assessed.

No significant differences were found for any of the subsets between patients with different *CFTR* genotypes or between exacerbating and clinically stable patients or between patients with or without *P. aeruginosa* or *S. aureus* infection.

Sex was correlated with both $CD16^{lo/neg}CD56^{int}$ NK cells (as % of NK cells) and dendritic cells (as % of $CD3^-$ cells), with higher levels of these subsets in women with CF compared with men. When NK cells were investigated as a whole rather than as individual subpopulations, they were found to be negatively correlated with age ($r = –0.51$, $P = 0.038$). However, when individual NK subpopulations were considered in relation to age, only $CD16^{hi}CD56^{int}$ NK cells, measured as either % of $CD3^-$ cells ($r = –0.60$, $P = 0.011$) or % of NK cells ($r = –0.60$, $P = 0.011$), were negatively correlated with age.

FEV1 % predicted was first analysed in all patients for whom spirometry and blood tests were performed within two weeks of each other (n = 13), in whom it was positively correlated with $CD16^{hi}CD56^{int}$ NK cells (as % NK cells) ($r = 0.65$, $P = 0.015$) and negatively correlated with $CD16^{lo/neg}CD56^{hi}$ NK cells (as % NK cells) ($r = –0.63$, $P = 0.021$). FEV1 % predicted was also positively correlated with $CD14^-$ MDSCs (as % $CD3^-$ cells) ($r = 0.60$, $P = 0.029$). Next, FEV1 % predicted was analysed in only clinically stable patients for whom spirometry and blood tests were performed within two weeks of each other (n = 9). When NK cells were considered as a whole population (% $CD3^-$ cells) they were positively correlated with FEV1 % predicted ($r = 0.77$, $P = 0.009$). However, when subpopulations of NK cells were investigated, varying relationships with lung function were identified. $CD16^{hi}CD56^{int}$ NK cells measured as either % $CD3^-$ cells ($r = 0.78$, $P = 0.007$) or % NK cells ($r = 0.76$, $P = 0.011$), and $CD16^{lo/neg}CD56^{int}$ (% $CD3^-$ cells) ($r = 0.78$, $P = 0.008$) were positively correlated with FEV1 % predicted, while $CD16^{lo/neg}CD56^{hi}$ NK cells (% NK cells) were negatively correlated with FEV1 % predicted ($r = –0.78$, $P = 0.008$). When MDSCs were considered as a whole population (% $CD3^-$ cells), they were positively associated with FEV1 % predicted ($r = 0.81$, $P = 0.005$). When subpopulations of MDSCs were analysed based on CD14 expression,

both $CD14^+$ and $CD14^-$ MDSCs (% $CD3^-$ cells) were positively correlated with FEV1 % predicted (r = 0.79, *P* = 0.006 and r = 0.89, *P* = 0.001, respectively).

Table 16. Correlations between monocyte subset percentages and clinical variables in CF patients

Parameter [no. of patients]	Monocytes (% $CD3^-$)	$CD16^{hi}CD14^{lo}$ Monocyte (% $CD3^-$)	$CD16^{hi}CD14^{lo}$ Monocyte (% monocytes)	$CD16^{int}CD14^{int}$ Monocyte (% $CD3^-$)	$CD16^{int}CD14^{int}$ Monocyte (% monocytes)	$CD16^{lo/neg}CD14^{hi}$ Monocyte (% $CD3^-$)	$CD16^{lo/neg}$ $CD14^{hi}$ Monocyte (% monocytes)
Age [17] (Spearman *r*)	0.34	0.04	–0.24	0.08	–0.16	0.36	0.16
***P* value**	[0.19]	[0.88]	[0.35]	[0.76]	[0.53]	[0.16]	[0.55]
FEV1 % pred. [13] (Spearman *r*)	–0.20	0.11	0.36	–0.15	–0.03	–0.15	–0.21
***P* value**	[0.51]	[0.72]	[0.23]	[0.63]	[0.93]	[0.62]	[0.49]
FEV1 % pred., stable patients only [9] (Spearman *r*)	–0.41	–0.04	0.47	–0.32	–0.08	–0.36	–0.26
***P* value**	[0.24]	[0.91]	[0.17]	[0.37]	[0.83]	[0.31]	[0.47]
Median percent for each subset							
Sex							
Male	57.40	4.80	7.84	1.49	2.62	51.55	89.60
Female	40.00	4.50	7.65	1.92	3.26	36.30	88.40
***P* value**	[0.15]	[0.62]	[0.96]	[0.31]	[0.25]	[0.20]	[0.72]
Genotype							
DF508 [8]	42.10	4.97	8.03	1.74	3.04	36.75	86.25
DF508 heterozygous/other [9]	46.00	4.50	7.65	1.53	3.26	40.80	89.10
***P* value**	[0.91]	[0.80]	[0.89]	[0.56]	[0.69]	[0.96]	[0.66]
Stable [14]	46.00	4.50	7.96	1.63	3.49	39.00	88.30
Exacerbating [7]	40.00	5.10	7.58	1.92	3.03	36.30	88.40
***P* value**	[0.76]	[0.79]	[0.80]	[0.81]	[0.99]	[0.81]	[0.73]
Chronic *P. aeruginosa* [10]	43.00	4.44	8.69	1.63	3.71	36.25	89.80
No chronic *P. aeruginosa* [7]	58.80	5.10	7.65	1.53	3.03	52.40	88.70
***P* value**	[0.72]	[0.98]	[0.96]	[0.98]	[0.65]	[0.54]	[0.69]
Current *S. aureus* infection [11]	46.00	5.10	8.26	1.83	3.26	40.80	88.40
No *S. aureus* infection [7]	39.40	2.47	6.26	1.39	3.38	36.00	91.10
***P* value**	[0.86]	[0.45]	[0.30]	[0.39]	[0.83]	[0.96]	[0.06]

[*P* values] (Mann–Whitney *U* test) indicating significant differences are shown in **bold.**

Table 17. Correlations between natural killer cell subset percentages and clinical variables in CF patients

Parameter [no. of patients]	Natural Killer Cells (% $CD3^-$)	$CD16^{hi}CD56^{int}$ Natural Killer Cell (% $CD3^-$)	$CD16^{hi}CD56^{int}$ Natural Killer Cell (% natural killer)	$CD16^{lo/neg}CD56^{hi}$ Natural Killer Cell (% $CD3^-$)	$CD16^{lo/neg}CD56^{hi}$ Natural Killer Cell (% natural killer)	$CD16^{lo/neg}CD56^{int}$ Natural Killer Cell (% $CD3^-$)	$CD16^{lo/neg}CD56^{int}$ Natural Killer Cell (% natural killer)
Age [17] (Spearman *r*)	–0.51	–0.60	–0.60	0.07	0.46	–0.13	0.23
***P* value**	**[0.038]**	**[0.011]**	**[0.011]**	[0.79]	[0.06]	[0.63]	[0.37]
FEV1% pred. [13] (Spearman *r*)	0.13	0.34	0.65	–0.51	–0.63	0.26	0.04
***P* value**	[0.67]	[0.26]	**[0.015]**	[0.07]	**[0.021]**	[0.38]	[0.90]
FEV1% pred. stable patients only [9] (Spearman *r*)	0.77	0.78	0.76	–0.41	–0.78	0.78	0.21
***P* value**	**[0.009]**	**[0.007]**	**[0.011]**	[0.24]	**[0.008]**	**[0.008]**	[0.56]
Median percent for each subset							
Sex							
Male	23.55	19.26	73.75	3.57	21.20	1.50	5.21
Female	23.10	18.20	78.60	2.56	10.60	2.44	8.68
***P* value**	[0.98]	[0.96]	[0.61]	[0.42]	[0.24]	[0.08]	**[0.03]**
Genotype							
DF508 [8]	15.95	12.21	74.90	2.93	16.20	2.14	9.07
DF508 heterozygous/other [9]	30.10	27.40	77.70	3.81	16.70	1.83	5.25
***P* value**	[0.47]	[0.32]	[0.54]	0.67	[0.96]	[0.37]	[0.06]
Stable [14]	29.45	25.20	76.70	2.56	12.80	1.83	6.07
Exacerbating [7]	15.20	12.10	79.35	2.36	15.50	1.84	7.29
***P* value**	[0.35]	[0.31]	[0.54]	[0.74]	[0.74]	[0.86]	[0.60]
Chronic *P. aeruginosa* [10]	21.40	16.35	74.90	2.94	16.20	1.84	6.97
No chronic *P. aeruginosa* [7]	30.10	25.20	77.70	3.29	16.70	2.00	6.00
***P* value**	[0.72]	[0.96]	[0.99]	[0.96]	[0.96]	[0.54]	[0.89]
Current *S. aureus* infection [11]	23.10	18.20	80.10	2.36	13.30	1.83	5.25
No *S. aureus* infection [7]	25.40	17.50	72.90	4.86	20.85	1.89	7.99
***P* value**	[0.64]	[0.96]	[0.12]	[0.26]	[0.59]	[0.59]	[0.18]

[*P* values] (Mann–Whitney *U*–test) indicating significant differences are shown in **bold**.

Table 18. Correlations between dendritic and myeloid derived suppressor cell subset percentages and clinical variables in CF patients

Parameter [no. of patients]	**Dendritic Cells (% CD3⁻)**	**MDSC (% CD3⁻)**	**CD14⁺ MDSC (% CD3⁻)**	**CD14⁺ MDSC (% MDSC)**	**CD14⁻ MDSC (% CD3⁻)**	**CD14⁻ MDSC (% MDSC)**
Age [17] (Spearman *r*)	0.04	–0.32	–0.31	–0.10	–0.36	0.06
***P* value**	[0.87]	[0.21]	[0.23]	[0.7]	[0.16]	[0.98]
FEV1 % pred. [13] (Spearman *r*)	0.80	0.51	0.51	–0.03	0.60	0.16
***P* value**	[0.08]	[0.08]	[0.07]	[0.93]	**[0.029]**	[0.71]
FEV1 % pred. stable patients only [9] (Spearman *r*)	0.20	0.81	0.79	–0.03	0.89	0.33
***P* value**	[0.58]	**[0.005]**	**[0.006]**	[0.93]	**[0.001]**	[0.35]
Median percent for each subset						
Sex						
Male	16.40	0.16	0.03	16.30	0.14	83.10
Female	22.10	0.49	0.10	14.40	0.42	83.50
***P* value**	**[0.02]**	[0.14]	[0.14]	[0.99]	[0.17]	[0.99]
Genotype						
DF508 [8]	21.65	0.37	0.04	13.90	0.31	84.95
DF508 heterozygous/other [9]	17.60	0.36	0.08	15.90	0.23	82.90
***P* value**	[0.24]	[0.89]	[0.74]	[0.54]	[0.74]	[0.81]
Stable [14]	26.70	0.45	0.03	12.50	0.32	85.10
Exacerbating [7]	35.00	0.36	0.06	22.20	0.23	77.50
***P* value**	[0.33]	[0.74]	[0.09]	[0.11]	[0.86]	[0.23]
Chronic *P. aeruginosa* [10]	18.50	0.22	0.03	15.90	0.18	81.85
No chronic *P. aeruginosa* [7]	20.30	0.49	0.10	13.30	0.42	86.70
***P* value**	[0.89]	[0.32]	[0.48]	[0.54]	[0.19]	[0.42]
Current *S. aureus* infection [11]	18.90	0.49	0.08	15.90	0.42	77.40
No *S. aureus* infection [7]	21.65	0.24	0.03	13.35	0.21	86.65
***P* value**	[0.88]	[0.40]	[0.40]	[0.53]	[0.35]	[0.26]

[*P* values] (Mann–Whitney *U* test) indicating significant differences are shown in **bold.**

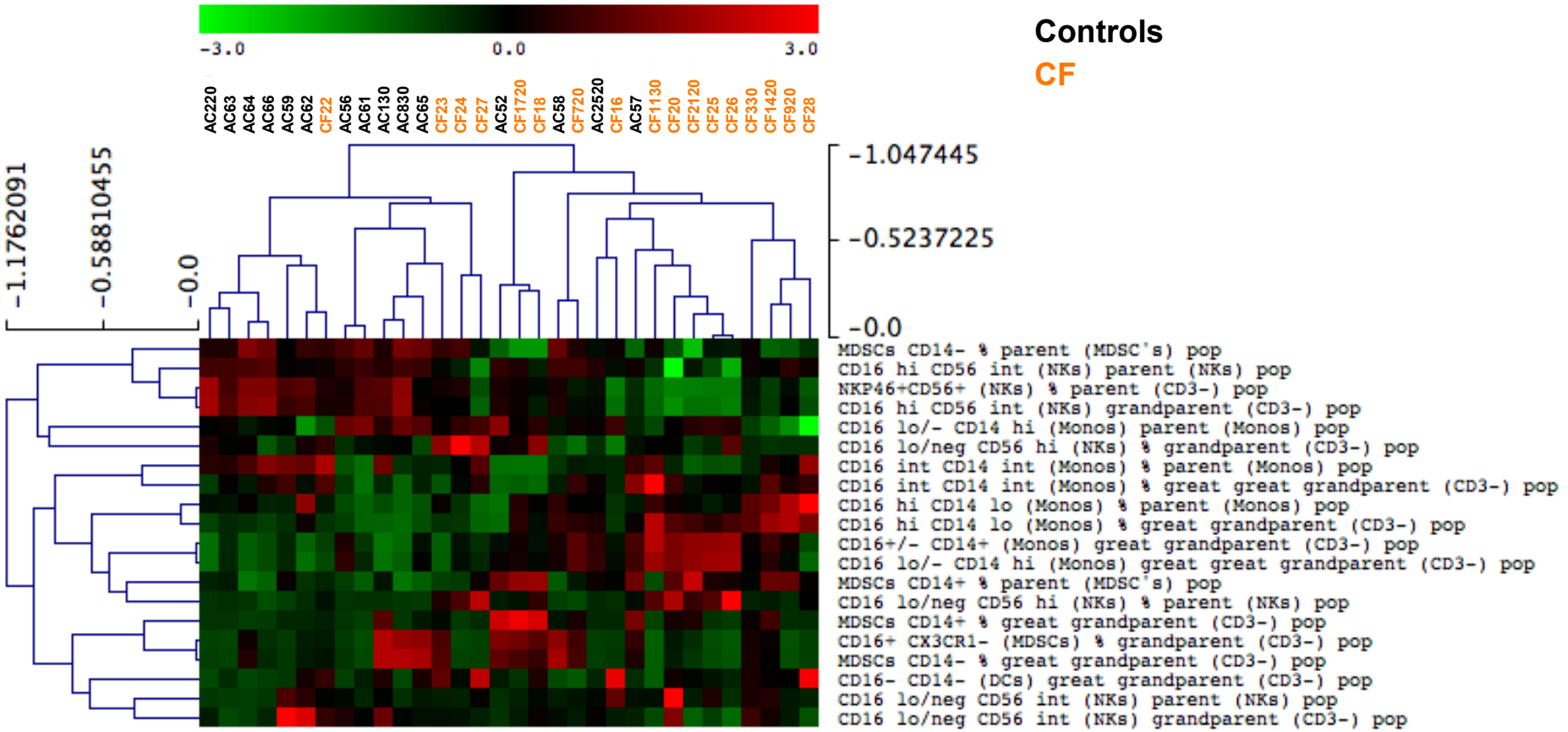
5.3.3 Multiexperiment Viewer (MeV) Bioinformatic Analysis of Peripheral Immune Cell Subsets

Flow cytometry is a high-throughput technology analysing multiple parameters for millions of cells, which produces large volumes of data. To improve the efficiency of analysis, bioinformatic analysis techniques using Multiexperiment Viewer (MeV) were employed. This allowed for visualisation of common trends/patterns between CF patients and healthy controls. Data were analysed using MeV as described in Chapter 2.

Firstly, all subsets detailed in Table 15 were analysed in all CF patients and controls. Although the results were not clear cut, a trend was seen towards separate clustering of CF patients and controls (Fig. 10).

Next, a heat map was generated that included only the subsets that differed significantly between controls and CF patients, to allow visualisation of patterns of upregulation/downregulation between the CF and control groups. (Fig. 11). This resulted in a clearer separation of the CF patients and controls, although, there were still two controls that clustered with the CF group and two CF patients that clustered with the control group (Fig. 12).

Lastly, all the subsets detailed in Table 15 were again analysed, but this time only including clinically stable CF patients compared with controls, in an attempt to identify changes that were not related to acute exacerbation of disease. A heat map was again generated including only the subsets that differed significantly between CF patients and controls, which showed a clear separation between clustering of CF patients and controls (Fig. 12), with only two control samples clustering amongst the CF group, and no patients clustering with controls.


-3.0
0.0
3.0
AC220
AC63
AC64
AC66
AC59
AC62
CF22
AC56
AC61
AC130
AC830
AC65
CF23
CF24
CF27
AC52
CF1720
CF18
AC58
CF720
AC2520
CF16
AC57
CF1130
CF20
CF2120
CF25
CF26
CF330
CF1420
CF920
CF28
-1.1762091
-0.58810455
-0.0
-1.047445
-0.5237225
-0.0
MDSCs CD14- % parent (MDSC's) pop
CD16 hi CD56 int (NKs) parent (NKs) pop
NKP46+CD56+ (NKs) % parent (CD3-) pop
CD16 hi CD56 int (NKs) grandparent (CD3-) pop
CD16 lo/- CD14 hi (Monos) parent (Monos) pop
CD16 lo/neg CD56 hi (NKs) % grandparent (CD3-) pop
CD16 int CD14 int (Monos) % parent (Monos) pop
CD16 int CD14 int (Monos) % great great grandparent (CD3-) pop
CD16 hi CD14 lo (Monos) % parent (Monos) pop
CD16 hi CD14 lo (Monos) % great grandparent (CD3-) pop
CD16+/- CD14+ (Monos) great grandparent (CD3-) pop
CD16 lo/- CD14 hi (Monos) great great grandparent (CD3-) pop
MDSCs CD14+ % parent (MDSC's) pop
CD16 lo/neg CD56 hi (NKs) % parent (NKs) pop
MDSCs CD14+ % great grandparent (CD3-) pop
CD16+ CX3CR1- (MDSCs) % grandparent (CD3-) pop
MDSCs CD14- % great grandparent (CD3-) pop
CD16- CD14- (DCs) great grandparent (CD3-) pop
CD16 lo/neg CD56 int (NKs) parent (NKs) pop
CD16 lo/neg CD56 int (NKs) grandparent (CD3-) pop

Controls

CF

Fig. 10. Heat map of monocyte, natural killer, dendritic and myeloid derived suppressor cell subset percentages in all CF patients and healthy controls. Raw data were imported in txt. file format to MeV. Data were normalised across rows and a nonparametric Kruskal–Wallis test was performed to determine differences in the proportions of each subset between groups. An unsupervised clustering analysis (hierarchical clustering) was performed using a nonparametric Spearman rank correlation distance metric and average linkage clustering. A heat map expression image was created to visualise results. Rows correspond to the immune cell subsets and columns correspond to individual subjects. Black squares indicate unchanged relative expression, green squares indicate down regulated relative expression and red squares indicate up regulated relative expression of that variable.

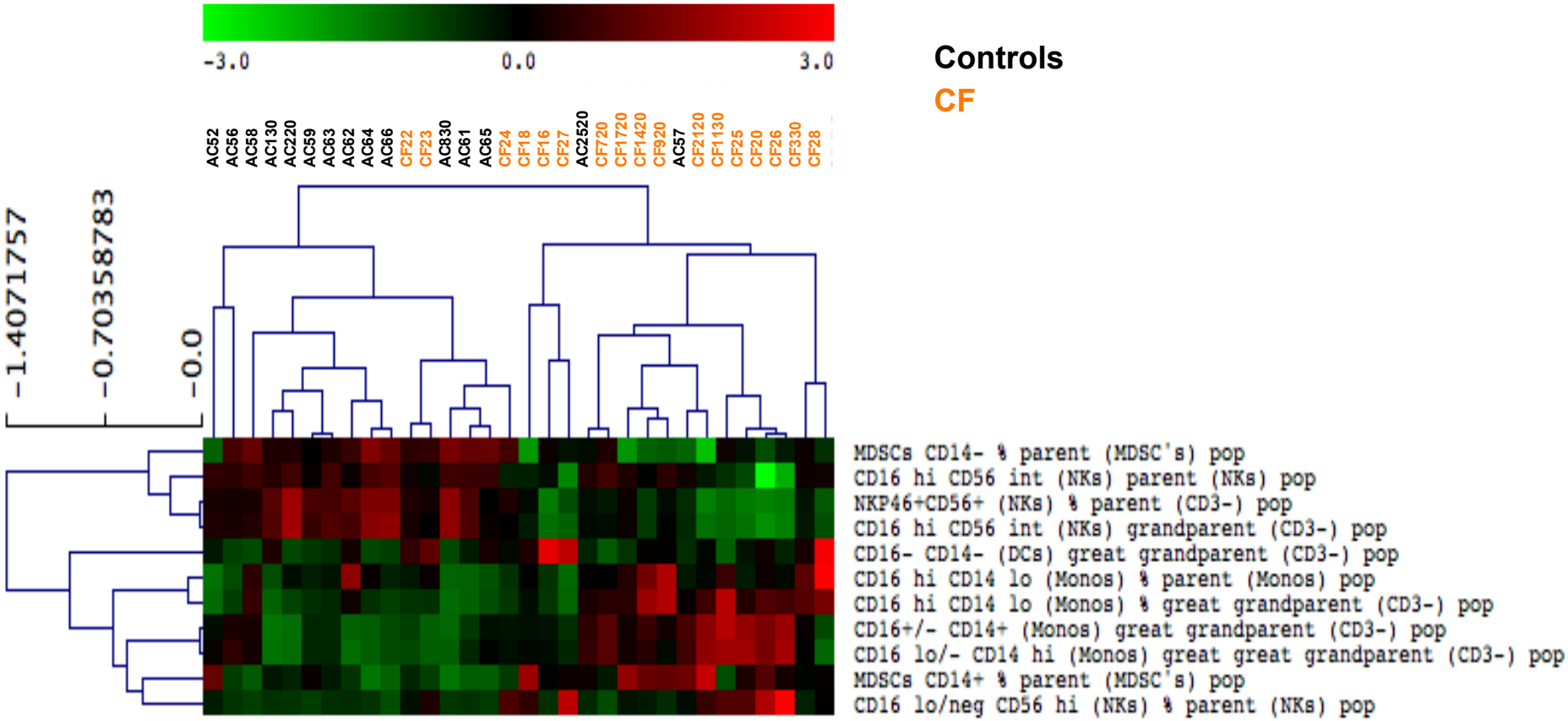
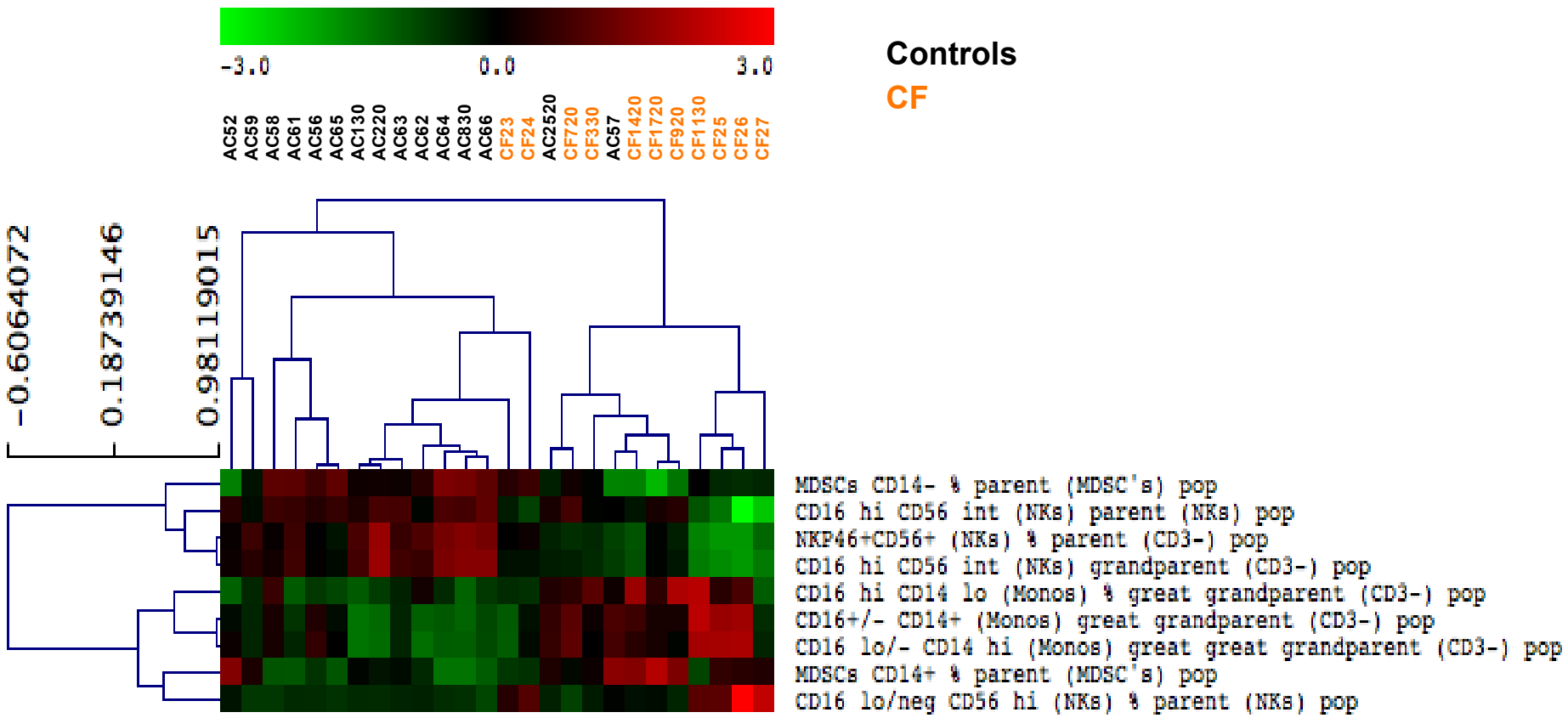

-3.0
0.0
3.0
Controls
CF
AC52
AC56
AC58
AC130
AC220
AC59
AC63
AC62
AC64
AC66
CF22
CF23
AC830
AC61
AC65
CF24
CF18
CF16
CF27
AC2520
CF720
CF1720
CF1420
CF920
AC57
CF2120
CF1130
CF25
CF20
CF26
CF330
CF28
-1.4071757
-0.70358783
-0.0
MDSCs CD14- % parent (MDSC's) pop
CD16 hi CD56 int (NKs) parent (NKs) pop
NKP46+CD56+ (NKs) % parent (CD3-) pop
CD16 hi CD56 int (NKs) grandparent (CD3-) pop
CD16- CD14- (DCs) great grandparent (CD3-) pop
CD16 hi CD14 lo (Monos) % parent (Monos) pop
CD16 hi CD14 lo (Monos) % great grandparent (CD3-) pop
CD16+/- CD14+ (Monos) great grandparent (CD3-) pop
CD16 lo/- CD14 hi (Monos) great great grandparent (CD3-) pop
MDSCs CD14+ % parent (MDSC's) pop
CD16 lo/neg CD56 hi (NKs) % parent (NKs) pop

Fig. 11. Heat map of significantly different monocyte, natural killer, dendritic and myeloid derived suppressor cell subset percentages between all CF patients and healthy controls. Raw data were imported in txt. file format to MeV. Data were normalised across rows and a nonparametric Kruskal–Wallis test was performed to determine differences in the proportions of each subset between groups. An unsupervised clustering analysis (hierarchical clustering) was performed using a nonparametric Spearman rank correlation distance metric and average linkage clustering. A heat map expression image was created to visualise results. Rows correspond to the immune cell subsets and columns correspond to individual subjects. Black squares indicate unchanged relative expression, green squares indicate down regulated relative expression and red squares indicate up regulated relative expression of that variable.


-3.0
0.0
3.0
AC52
AC59
AC58
AC61
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AC65
AC130
AC220
AC63
AC62
AC64
AC830
AC66
CF23
CF24
AC2520
CF720
CF330
AC57
CF1420
CF1720
CF920
CF1130
CF25
CF26
CF27
-0.6064072
0.18739146
0.98119015
Controls
CF
MDSCs CD14- % parent (MDSC's) pop
CD16 hi CD56 int (NKs) parent (NKs) pop
NKP46+CD56+ (NKs) % parent (CD3-) pop
CD16 hi CD56 int (NKs) grandparent (CD3-) pop
CD16 hi CD14 lo (Monos) % great grandparent (CD3-) pop
CD16+/- CD14+ (Monos) great grandparent (CD3-) pop
CD16 lo/- CD14 hi (Monos) great great grandparent (CD3-) pop
MDSCs CD14+ % parent (MDSC's) pop
CD16 lo/neg CD56 hi (NKs) % parent (NKs) pop

Fig. 12. Heat map of monocyte, natural killer, dendritic and myeloid derived suppressor cell subset percentages in clinically stable CF patients and healthy controls. Raw data were imported in txt. file format to MeV. Data were normalised across rows and a nonparametric Kruskal–Wallis test was performed to determine differences in the proportions of each subset between groups. An unsupervised clustering analysis (hierarchical clustering) was performed using a nonparametric Spearman rank correlation distance metric and average linkage clustering. A heat map expression image was created to visualise results. Rows correspond to the immune cell subsets and columns correspond to individual subjects. Black squares indicate unchanged relative expression, green squares indicate down regulated relative expression and red squares indicate up regulated relative expression of that variable.

5.4 Discussion

This study shows for the first time that proportions of monocytes, DCs, NK cells and MDSC are all altered in CF peripheral blood compared with healthy controls. We also showed that in CF patients, peripheral total and subpopulations of NK and MDSCs were correlated with lung function as assessed by FEV1 % predicted.

Previous studies of NK cells have been undertaken primarily in disease states such as cancer, tumours, autoimmunity, viral infections and asthma (313), with changes in not only numbers but also function being implicated in disease pathogenesis. There is very little research investigating NK cells in CF. The three studies investigating this cell type in CF (138-140) have all shown peripheral blood decreases, and one (138) also demonstrated a positive correlation between NK cells and FEV1 % predicted. However, all of these studies investigated these cells as one heterogeneous population rather than focussing on individual NK subsets.

Our data confirmed that NK cells, measured as a single population based on NKp46 and CD56 expression, are decreased in CF peripheral blood compared with that of healthy controls. Because this innate population is involved in the initial response to infection, particularly viral infection, this may have a negative impact on the ability of CF patients to deal with viruses. Unfortunately, as screening for viruses is not included in routine clinical tests we do not have data pertaining to viral infections in the CF patients.

Further analysis of NK subpopulations using CD56 and CD16 expression revealed a shift in CF patients from $CD56^{int}$ to $CD56^{hi}$ NKs, with a positive correlation between $CD56^{int}$ NKs and FEV1 % predicted and a negative correlation between $CD56^{hi}$ NKs and FEV1 % predicted. These subpopulations of NK cells are functionally different: $CD56^{int}$ cells are highly cytotoxic while $CD56^{hi}$ cells have a strong propensity for cytokine production. Therefore, decreased $CD56^{int}$ (cytotoxic) NK cells could contribute to difficulty in limiting the scope of viral infections because of decreased killing of virus-infected cells, while increased $CD56^{hi}$ (cytokine producing) cells could increase tissue damage caused by high levels of cytokine production. The negative correlation seen between the $CD56^{hi}$ cytokine-producing NK cells and FEV1 % predicted could be related to the damaging effects on lung tissue of the key proinflammatory cytokines that they produce, IFNγ and TNF, especially because $CD56^{hi}$ NK cells are known to be the dominant NK cell subset in tissues such as the lung (136). However, previous studies have also shown that $CD56^{hi}$ NK cells are able to

produce the Th2-associated cytokines, IL-4, IL-5 and IL-13 when stimulated *ex vivo*, and that this cytokine production is promoted by the presence of IL-4 and inhibited by the presence of IL-12 and IL-13 (136, 314-316). Given that there is a known Th2 dominance in CF lungs and that Th2-dominated immune responses have been associated with poorer lung function, it would be reasonable to suggest that if the $CD56^{hi}$ NK cells were producing the Th2-associated cytokines, they too would be negatively associated with lung function. Consistent with this hypothesis, NK cells producing Th2-associated cytokines have been implicated in the pathogenesis of asthma, another Th2-dominated inflammatory disease (317). The positive correlation seen between $CD56^{int}$ cytotoxic NK cells and FEV1 % predicted could be because of the ability of these cells to kill virus-infected cells and thus reduce the spread of infection and damage to the lungs.

It is not possible to determine from our data whether the decrease in CF peripheral NK cells is because of the homing of these cells to the lungs during lung inflammation and infection, as has been reported previously (136, 318-324), or is because of an absolute decrease in their numbers, as seen in autoimmune conditions such as systemic lupus erythematosus (SLE) (325). However, our findings are similar to those seen in SLE, which also presents with a decrease in peripheral NK cells and a switch from $CD56^{int}$ to $CD56^{hi}$ NK cells. Autoreactive T cells are also known to mediate NK cell degeneration in autoimmune disease (326). This may suggest that there are similar autoimmune mechanisms contributing to CF pathogenesis. This is further supported by the results reported by Lachenal *et al.* (327) who demonstrated a high frequency of autoantibodies in CF that were associated with the severity of lung disease and prognosis. The mechanism behind autoimmunity in CF is not yet understood with the possibility, as suggested by Lachenal *et al.* (327), that the immune system responds nonspecifically because of chronic infection and inflammation, and that autoimmunity could be contributing to the hyperinflammation seen in CF and promoting infection and lung damage.

Alternatively, or in addition to this, the effects of *P. aeruginosa*, the dominant CF pathogen, on NK cells could be contributing to their decreased levels. One study showed that *P. aeruginosa* is able to kill NK cells through induction of apoptosis (328), while Broquet *et al.* reported that a systemic depletion of NK cells increased the susceptibility to *P. aeruginosa* infection in a mouse model of *P. aeruginosa* pneumonia (329). This mouse model also presented with an influx of neutrophils that was associated with this reduction in NK cells; a similar manifestation is seen in CF. It has also been shown, using a mouse model, that NK

cell-depleted mice are more susceptible to pulmonary infection with *S. aureus* and develop a pulmonary expansion of neutrophils and macrophages in response to NK cell reduction, which also resembles features of CF. In addition, cytokines such as IL-12 and IL-18, involved in the initiation of cell mediated immunity through Th1 cell induction, are also required for the generation of NK cells and have been reported to be low in CF lungs and peripheral blood (249, 330, 331).

Another innate immune cell population of interest, given its recent implication in many disease pathologies, particularly infection and inflammation, is MDSCs. Because MDSCs are a newly described and heterogeneous population of immature myeloid cells, their characterisation is ongoing. However, two distinct subsets have been identified, known as monocytic MDSCs and granulocytic MDSCs (120). Our flow cytometry antibody panel identified monocytic and granulocytic MDSCs based on CD11b, CD16 and CD14 expression. There is only one study, by Rieber and colleagues (126), describing MDSCs in CF, which focussed on granulocytic MDSCs in CF patients with and without *P. aeruginosa* infection compared with healthy subjects (126), and found that granulocytic MDSCs were positively correlated with lung function and were directly able to downregulate several cytokines (IFNγ, IL-13, IL-6, GM-CSF, IL-10) including IL-17. Consistent with this, we also showed a positive correlation between the level of granulocytic MDSCs and FEV1 % predicted. However, Rieber *et al.* (126) reported that granulocytic MDSCs were increased in CF patients chronically infected with *P. aeruginosa* compared with healthy controls, which contrasts with our finding of decreased granulocytic MDSCs in CF, even in only those positive for *P. aeruginosa*. This discrepancy cannot be explained, but may be accounted for by differences in the method of granulocytic MDSC identification, with Rieber and colleagues using a much more extensive array of markers such as CD33, CD66b, IL-4Rα and HLA-DR. Given the relationship Rieber and colleagues have shown between granulocytic MDSCs and Th17 cells, it would be expected that a decrease in granulocytic MDSCs would result in an increase of Th17 levels. As we have previously shown no change in peripheral Th17 levels this may suggest that monocytic MDSCs, which Rieber and colleagues did not investigate, may also be capable of suppressing Th17 cell proliferation. This is supported by our finding that monocytic MDSCs are also positively correlated with FEV1 % predicted in CF. With regard to the positive association we saw between MDSCs and FEV1 % predicted, Rieber *et al.* (126) suggested that *P. aeruginosa* is able to induce MDSCs that are in turn able to downregulate Th17 responses, thereby reducing IL-17 production and the associated

recruitment of damaging neutrophils. This may explain why we did not observe an increase in Th17 levels in peripheral blood, as has been shown in the lungs, because MDSCs measured as one heterogeneous population are unchanged in CF and are therefore able to keep Th17 cells under control.

While we saw no difference in the proportion of MDSCs between CF and controls, there is a switch from a granulocytic to a monocytic MDSC phenotype in CF. The upregulation of CD14 on MDSCs may be because of the presence of chronic bacterial infection in people with CF, because CD14 is a coreceptor for detection of bacterial LPS.

Given their association with FEV1 % predicted, NK cells and MSDC could potentially be considered useful therapeutic targets, and as suggested previously for peripheral blood T cells (310), act as potential surrogate markers of lung function.

Other innate immune populations that have a major influence on the adaptive immune response are antigen presenting cells such as monocytes and DCs. These cells are of particular interest because of their ability to directly influence T cell responses, which are known to be dysregulated in CF, and because both monocytes and DCs have been reported to normally express CFTR (23, 311). While CF monocytes have been reported to have defects in adhesion (85), secretion of IL-8, proteinase and elastase (88-90), and complement-mediated phagocytosis of pathogens such as *P. aeruginosa* (92), these studies have all investigated monocytes as one heterogeneous population.

We have shown an overall increase in peripheral blood monocytes in CF with expansion of both the 'classical' ($CD16^{lo/neg}CD14^{hi}$) and 'non-classical' ($CD16^{hi}CD14^{lo}$) monocyte populations. The levels of these cells we recorded in healthy controls were within the range reported in the published literature (74). A contributing factor to this expansion, given that CF is characterised by a Th2-dominated immune response, could be the Th2 cytokine IL-4 that is increased in CF peripheral blood (301), which is known to drive expansion of monocytes during Th2 inflammation (332). Classical and non-classical monocytes are able to migrate to sites of infection and inflammation via the chemotactic effects of the chemokines CCL2 and CX3CL1, respectively (333). Increases in peripheral blood and sputum CCL2 and normal levels of peripheral blood CX3CL1 have been reported in CF patients (244, 334). Therefore, it would be expected that increased migration of classical monocytes to the lungs would occur in CF because of their high expression of CCR2, and consequently a reduction would be seen in the periphery. However, given the chronic nature of lung infection and inflammation in CF,

it is possible that over time, a high level of these cells also becomes detectable in the periphery. Because this population is considered proinflammatory, with the ability to develop into both lung macrophages and DCs during inflammation, persistently increased levels of these cells and their subsequent trafficking to the lung could be considered harmful to lung tissue.

Although an increase in non-classical monocytes has been reported during several types of bacterial infections (74, 335), their role, positive or negative, during these infections is yet to be elucidated. Non-classical monocytes have been shown to be potent stimulators of IL-17 production by T cells (336) and therefore, in CF, expansion of non-classical monocytes could be contributing to disease pathogenesis by increasing Th17 differentiation. Although this was not detectable in the periphery because we have shown normal levels of Th17, the high Th17 reported in the CF lung (53, 263, 283) may be as a result of these increased non-classical monocytes that are known to home to inflamed tissues (76). In addition to this, this population has been shown to preferentially differentiate into alternatively activated 'M2' macrophages (337), particularly in response to the Th2-associated cytokines IL-4 and IL-13, which in CF have been shown to be associated with poorer lung function (103).

Only one previous study has investigated subsets of peripheral monocytes in CF (244), which characterised only two subpopulations based on CD16 and CD14 expression, rather than the now commonly accepted three subsets, because it included the 'intermediate' ($CD16^{int}CD14^{int}$) population within the 'classical' ($CD16^{lo/neg}CD14^{hi}$) and 'non-classical' ($CD16^{hi}$ $CD14^{lo}$) populations. Although that study reported an increase in CCL2, a monocyte chemoattractant, in the peripheral blood of CF patients, it did not show any changes in the levels of the two monocyte populations. However, the study was performed in a much younger cohort than that analysed in our study, which could explain the contrasting results if the changes are associated with chronic infection and disease progression.

Despite this possibility, the changes in classical and non-classical monocyte subsets in peripheral blood that we observed were not associated with any clinical variable we analysed, including FEV1 % predicted. Understanding the role of individual monocyte subpopulations in disease states is an emerging area of research, with controversy still surrounding the functional roles of each subset, as discussed in section 2.2.1. While we and others have demonstrated an expansion of monocytes in CF peripheral blood, more detailed analyses of the function of individual subsets in CF are required to determine their specific role, if any, in CF pathogenesis.

DCs are particularly important during immune responses because they are uniquely able to present antigen to and stimulate naïve T cells. We have shown for the first time that there is an increase in peripheral blood DCs in CF patients. Our method of DC identification did not allow us to differentiate between specific dendritic cell subsets, because it was based upon the lack of expression of lineage-specific markers such as CD3, NKP46, CD56, CD14 and CD16. During infection, DCs home towards the lymph nodes to present processed antigen to T cells to elicit an immune response against invading pathogens. It would therefore be expected that peripheral DC levels would be decreased in CF because of the presence of chronic lung infection. However, under inflammatory conditions classical monocytes, which we have also shown to be increased, are able to differentiate into DCs, which may explain the observed increase in peripheral blood DCs.

Many of the studies investigating DCs in CF use mouse models, and have demonstrated a downregulation of costimulatory molecules CD40, CD80 and CD86, reduced maturation capacity and reduced expression of genes associated with membrane structure and lipid metabolism in DCs (23, 114-118), particularly in response to specific pathogens such as *P. aeruginosa* (23, 117) and *B. cepacia* complex (118). However, the role of DCs in CF lung disease has not been fully elucidated. In a mouse model of *P. aeruginosa* infection, DCs were found to be involved in the polarisation of T cells towards a Th2 phenotype (330), which may suggest that increased levels of DCs could be contributing to the detrimental Th2 bias in CF. It has also been shown using a mouse model that after exposure to fungal antigens, DCs experience an influx of Ca^{2+} that allows production of IL-2, which in turn controls Th17 responses (338). Because defective CFTR is known to affect Ca^{2+} signalling in CF cells, it could be suggested that increased levels of DCs with an altered capacity to control Th17 differentiation may also be contributing to CF pathogenesis. Further studies investigating human DC populations are required to confirm whether similar events are occurring in people with CF. However, because, as was seen for monocytes, altered DC proportions did not correlate with any clinical parameters analysed including FEV1 % predicted, they may not directly influence CF disease pathology.

Analysis of such a wide range of immune cell subsets produced a large amount of data, meaning that bioinformatic techniques were particularly useful to assist the analysis. This analysis of these data allowed the identification and visualisation of patterns of changes in the immune cells of interest. Firstly, identification of relationships between study participants was possible using hierarchical clustering tools, which identified a clear divide between CF

patients and healthy controls. This discrete clustering of the CF and healthy control groups was most distinct when only clinically stable patients were included in the analysis, suggesting a distinct innate immune cell phenotype in CF patients compared with healthy controls. Secondly, it allowed the identification of relationships between individual cell populations and consequently, whether changes in these populations could be associated. For instance, the shifts toward $CD14^{+}$ MDSCs and $CD56^{hi}$ (cytotoxic) NK cells were linked, possibly indicating that these both change in response to infection, given that CD14 is a coreceptor for detection of bacterial LPS and $CD56^{hi}$ NKs produce inflammatory cytokines. Interestingly, we found that changes in classical monocytes and intermediate monocytes were more closely related to each other than to those in non-classical monocytes. This is in contrast to the ongoing debate suggesting that intermediate monocytes are more closely linked to non-classical monocytes (74) than classical monocytes.

The main strength of this study is that it investigated a wide range of peripheral innate immune cells and their subsets within one analysis. While we did not include neutrophils in our analyses this population has been extensively studied in CF. Our study also investigated only the proportions of these immune cells and did not assess their functions. Understanding function in addition to proportion will provide a more detailed picture of CF innate immune dysfunction. Also, while investigating these cells in the periphery has its advantages, also assessing their levels in the lungs and lymph nodes would provide a more complete picture.

In conclusion, this study has shown decreased peripheral levels of NK cells with a switch from a $CD56^{int}$ to $CD56^{hi}$ phenotype, an increase in classical ($CD16^{lo/neg}CD14^{hi}$) and non-classical ($CD16^{hi}$ $CD14^{lo}$) monocytes, an increase in dendritic cells and lastly a switch from $CD14^{-}$ granulocytic to $CD14^{+}$ monocytic MDSCs. A negative correlation was also seen between $CD56^{hi}$ NK cells and FEV1 % predicted while a positive correlation was seen between $CD56^{int}$ NK, $CD14^{+}$ MDSCs, $CD14^{-}$ MDSCs and FEV1 % predicted. This provides new information about innate immune cells in CF, and the bioinformatic analysis also allowed the identification of a broad CF immune cell phenotype. This study demonstrates again that peripheral immune cell subsets can reflect lung pathology in CF. However, further research is required to determine any prognostic value of these measurements in predicting the progression of CF-related lung disease. NK and MDSC subsets, given their relationship with FEV1 % predicted, may also prove to be useful therapeutic targets, although further studies are required to confirm this. Given the widespread dysregulation of the innate immune response in CF, correction of this could, in addition to the traditional lung-based outcomes

such as increased lung function, reduced exacerbation and infection, also be an important marker of the efficacy of new CF therapeutics.

CHAPTER 6

RT–qPCR Analysis of Peripheral Blood $CD4^+$ T Cell Subset Related Markers

6.1 Introduction

Cystic fibrosis is characterized primarily by chronic infection and inflammation of the lungs but the role of the immune system, particularly the adaptive immune response, is yet to be fully elucidated. Dysfunctional *CFTR* has been shown to directly lead to aberrant T cell responses in CF (22) via increased nuclear localization of the transcription factor NFAT, which leads to increased production of Th2-assoiciated cytokines. Because NFAT activation has been shown to be altered as a result of dysfunctional CFTR and because NFAT is associated with other T cell subset transcriptional regulators (339), the possibility arises that transcriptional regulation of T cell function may be affected at many points. The CF immune response is also known to be hyperinflammatory, but the contribution of $CD4^+$ T cells to this state is as yet unclear.

Investigation of transcription factors at a cellular level is problematic because the proteins are generally expressed at very low levels. The most practical approach for assessing their role in $CD4^+$ T cell dysregulation is through analysis of gene expression, which provides much greater sensitivity and allows for detection of a large range of markers. While differentiation of $CD4^+$ T cells into specific subsets is controlled by many transcriptional regulators, many subsets have a primary regulator (see Figure 1). In order to investigate transcriptional regulation of $CD4^+$ T cell subsets we used RT–qPCR to analyse the level of expression of the transcription factors *T-BET*, *GATA3*, *c-REL*, *FOXP3*, *STAT3*, *c-MAF* and *AHR* in isolated $CD4^+$ T cells. This allowed identification of transcription factors involved in differentiation of Th1, Th2, Th17, nTreg, iTreg, IL-10^+ Tr1, and TGFβ^+ Th3 cells, respectively.

To investigate the role of $CD4^+$ T cells in the hyperinflammatory response we also investigated gene expression of inflammatory and anti-inflammatory markers such as TNF, IL-6 and peroxisome proliferator activated receptor (PPAR)γ. TNF and IL-6 are proinflammatory cytokines while PPARγ is an anti-inflammatory transcription factor. Paraoxonase 2 (PON2), an enzyme that can degrade a key *P. aeruginosa* signalling molecule and expression of which is known to be correlated with that of PPARγ in BAL cells of CF patients (340), was also investigated.

Lastly, we have previously shown alterations in the levels of the homing markers, CXCR3, CCR4 and CCR6, on $CD4^+$ and Treg cells using flow cytometry. We therefore also assessed

the level of gene expression of these markers to further clarify whether dysregulation of these markers is present at the mRNA level.

6.2 Materials and Methods

6.2.1 Isolation of $CD4^+$ T Cells from PBMC

Fresh and frozen PBMC were used for isolation of $CD4^+$ T cells. $CD4^+$ cells were isolated using the EasySep Human $CD4^+$ T Cell Isolation Kit (Stemcell Technologies, Tullamarine, VIC, Australia) according to the manufacturer's instructions. Briefly, cells were suspended in a 12 × 15 mm polystyrene tube at a concentration of 5×10^7 cells/mL in cell sorting medium containing PBS, 2% FCS and 1mM EDTA. Cells were then treated with 100 μg/mL DNase (Stemcell Technologies) for 15 min at room temperature. EasySep Human $CD4^+$ T cell enrichment cocktail was added at 50 μL/mL for 10 min at room temperature followed by EasySep D Magnetic Particles at 100 μg/mL for 5 min at room temperature. The cell suspension volume was increased to 2.5 mL with cell sorting medium, mixed well, and then placed in an EasySep Magnet for 5 min at room temperature. The negatively selected $CD4^+$ cells were then decanted ready for use. The purity of the $CD4^+$ cells ranged between 93–97%, and a representative example of the results is shown in Figure 13.

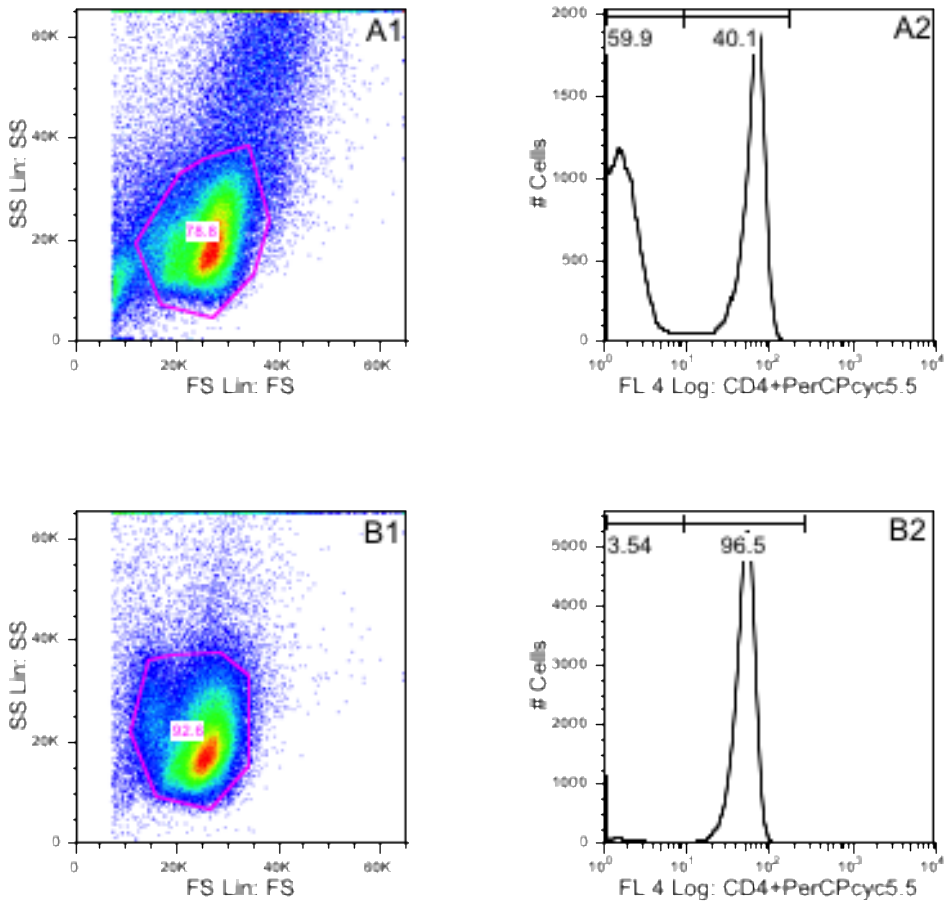


Fig. 13. Histograms illustrating CD4$^+$ cell purity obtained using the CD4$^+$ isolation technique. 'A1' and 'B1' shows gating of the main cell populations of unsorted and CD4$^+$ sorted PBMC respectively. 'A2' and 'B2' histograms show percent of CD4 positive and negative cells in unsorted and CD4$^+$ sorted cells respectively. Original data.

6.2.2 RNA Extraction and DNase Treatment

RNA was extracted using the ReliaPrep RNA Cell Miniprep System (Promega, Alexandria, NSW, Australia) according to the manufacturer's instructions. Briefly, $5 \times 10^5 - 2 \times 10^6$ cells were washed with 1× PBS at 300 × *g* for 5 min. To the cell pellet 250 μL BL + TG buffer (as detailed in appendix 1.4) was added, the tube vortexed and left for 3 min. Then, 85 μL of isopropanol was added, tube vortexed, and the sample was transferred to a ReliaPrep Mini Column. The column was centrifuged at maximum speed (20,800 × *g)* for 1 min at room temperature and supernatant discarded. Bound RNA was washed with 500 μL RNA Wash Solution, column centrifuged at maximum speed for 1 min at room temperature and the wash solution discarded, after which 30 μL DNAse I mixture was added directly to the membrane and column incubated for 15 min at room temperature. Column was then washed successively with 200 μL Column Wash Solution, 500 μL RNA Wash Solution, 500 μL RNA Wash Solution. RNA was eluted in water by centrifuging at maximum speed at room temperature. The eluted RNA was transferred onto the membrane and column re-centrifuged to increase the concentration of RNA released from the column.

6.2.3 cDNA Synthesis

cDNA was synthesised using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Castle Hill, NSW, Australia) according to the manufacturer's instructions. Briefly, a 10 μL reaction was prepared containing 1 μL of anchored-oligo$(dT)_{18}$, primer (50 pmol/μL), water and DNase treated RNA (172.8 ng). The template–primer mixture was denatured by heating in a thermal block cycler with a heated lid at 65 °C for 10 min. The reaction was made up to 20 μL by the addition of 4 μL of Transcriptor Reverse Transcriptase Reaction Buffer (5× concentrate), 0.5 μL of Protector RNase Inhibitor (40 U/μL), 2 μL of deoxynucleotide mix (10 mM each) and 0.5 μL of Transcriptor Reverse Transcriptase (20 U/μL). The 20 μL reaction was incubated in a thermal block cycler with heated lid at 50 °C for 60 min and the Transcriptor Reverse Transcriptase subsequently inactivated by heating at 85 °C for 5 min. The reaction was cooled to 4 °C and samples were stored at –80 °C.

6.2.4 Primer Design

All primers, except for those used to amplify AHR, were either designed using Primer 4 based on available sequences (http://www.ncbi.nlm.nih.gov) or were previously published and were synthesised by Geneworks (Thebarton, SA, Australia). The primers targeting AHR were designed and synthesised by Qiagen (Melbourne, VIC, Australia). Primers used in this study are detailed in Table 19. Primer specificity was confirmed via melt curve analysis and products run on agarose gels to ensure correct band size.

Table 19. Primer sequences for RT–qPCR analysis.

Target	Role	Forward Primer	Reverse Primer	Source
RPL19	Reference gene	GCGGATTCTCATGGAACACA	GGTCAGCCAGGAGCTTCTTG	Jubb *et al.* (341)
T-BET	Major transcriptional regulator of Th1 lineage	GATGCGCCAGGAAGTTTCAT	GCACAATCATCTGGGTCACATT	Vale-Pereira *et al.* (342)
GATA3	Major transcriptional regulator of Th2 lineage	TCCTGTGCGAACTGTCA	TCTGGTCTGGATGCCTT	Refaat *et al.* (343)
c-MAF	Major transcriptional regulator of Tr1 lineage Involved in IL-4 production	CCGCATCATCAGCCA	GCGCGTAGCCATCGA	Li *et al.* (344)
c-REL	Major transcriptional regulator of nTreg lineage Involved in IL-2, IL-3, IFNγ and GM-CSF production by T cells	ATCATGCCTCAATGG	TTGTATTGCCTGAGC	This study
AHR	Major transcriptional regulator of Tr1 lineage and iTreg Involved in Treg/Th17 balance	Sequence unknown	Sequence unknown	Qiagen
FOXP3	Provides cells with suppressive function Marker of T regulatory cells	GAAACAGCACATTCC	ATGGCCCAGCGGATG	Burgler *et al.* (345)
STAT3	Major transcriptional regulator of Th17 lineage	GACGTGTCTGGTTGAGCTCAGG	CACAGAAACTCTGATCAGCTGAGG	This study
PPARg	Anti-inflammatory transcription factor	AGCTGAACCACCCTGAGTCC	TCATGTCTGTCTCCGTCTTCTTG	Griffin *et al.* (340)
PON2	Degrades a key *P. aeruginosa* signalling molecule	GCCAACAATGGGTCTGTTCTCC	CAGCTTCCCATCATACACTGAGGC	Griffin *et al.* (340)
TNF	Inflammation	GGTGCCATCAGAGGG	GGGCAGCCTTGGCCC	This study
IL-6	Inflammation	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC	This study
CXCR3	Chemokine receptor preferentially expressed on Th1 cells	CTCCACCTAGCTGTAGCAGA	AGGAAGATGAAGTCTGGGAG	This study
CCR4	Chemokine receptor preferentially expressed on Th2 cells	GAAGAAGAACAAGGCGGTGAAGAT	ATGGTGGACTGCGTGTAAGATGAG	This study
CCR6	Chemokine receptor preferentially expressed on Th17 cells	GCCATCCGTGTAATCATAGCTGTG	CGTTATCTGCGGTCTCACTGGTC	This study

6.2.5 RT–qPCR Data Acquisition

RT–qPCR data acquisition was performed using a Light Cycler 480 (Roche Diagnostics, Castle Hill, NSW, Australia). The conditions used for the Light Cycler 480 analysis are listed in Table 20.

Table 20. Roche Light Cycler 480 run conditions for RT–qPCR analysis.

Program	Cycles	Analysis Mode	Target (°C)	Acquisition mode	Hold	Ramp Rate	Acquisition
Denature	1	None	95	–	5 min	4.8	–
PCR	40	Quantification	95	–	10 s	4.8	–
			60	–	10 s	2.5	–
			72	Single	30 s	4.8	–
Melt	1	Melting Curves	95	–	10 s	4.8	–
			58	–	1 s	2.5	–
			95	Continuous	–	0.11	5
Cool	1	None	40	–	5 min	2.5	–

6.2.6 Data Analysis

Data analysis was performed on the LightCycler480 using the second derivative maximum method. From the extracted data, mRNA expression was calculated relative to the reference gene RPL19 which is commonly used for gene expression analysis in CD4 T cells (346, 347). Relative mRNA was calculated using the following formula developed by Livak and Schmittgen (348):

$$\frac{\text{PCR efficiency of target gene (CT control sample – CT patient sample)}}{\text{PCR efficiency of reference gene (CT control sample – CT patient sample)}}$$

All data were analysed using GraphPad Prism Version 6 (GraphPad Software). Nonparametric tests (Mann–Whitney *U* and Kruskal–Wallis) were applied to the data to investigate the differences between groups. To determine correlations between clinical parameters and subset percentages a nonparametric Spearman correlation was performed. All *P* values < 0.05 were considered significant.

6.3 Results

6.3.1 Comparison between CF and Control Groups

There was no significant difference in median age or sex distribution between the control and CF groups. The comparison of the expression of mRNA for T cell subset and inflammation related markers relative to the reference gene *RPL19* is shown in Table 21. *TNF* expression was increased (P = 0.023) while *c-REL* (required for nTreg differentiation) and *T-BET* (required for Th1 differentiation) expression were decreased (P = 0.006 and 0.017 respectively) in CF patients compared with healthy controls. There were no differences between CF patients and healthy controls in the expression of *PPARγ, PON2, CCR4, CCR6, IL-6, FOXP3, GATA3, STAT3* or *AHR* although *CXCR3* (Th1-associated homing marker) and *c-MAF* (required for Tr1 differentiation) were trending towards being decreased in CF compared with healthy controls (P = 0.08 and 0.06, respectively).

6.3.2 Comparisons within the CF Cohort

We next investigated any important associations between the expression of the measured genes and clinical parameters within the CF group, as described in Chapter 2. Investigating those with (n = 3) or without (n = 15) current *A. fumigatus* infection and those with (n = 16) or without (n = 1) current treatment with any antimicrobial was not possible given the small sizes of one of the groups. No associations were found between the expression of any of the measured genes and any clinical parameters. However, expression of many of the genes investigated were found to be correlated with each other, as indicated in Table 22, with the exception of *c-REL* (required for nTreg differentiation) and *c-MAF* (required for Tr1 differentiation), which appeared to be quite independent.

Table 21. Comparison of the relative mRNA expression of immune-related markers in $CD4^+$ cells in control and CF groups

	Control	CF	*P* value
N	16	10	
Age median (range)	28 (23–54)	25.5 (19–55)	0.32
Sex male (%)	6 (37.5%)	5 (50%)	
Relative mRNA Expression			
TNF median (range)	1.6050 (0.7819–6.3420)	3.0770 (0.9395–11.2000)	**0.023**
PPARγ median (range)	0.0321 (0.0094–0.1012)	0.0611 (0.0030–0.2449)	0.33
PON2 median (range)	0.1024 (0.0439–0.2432)	0.1253 (0.0127–0.5783)	0.48
CCR4 median (range)	0.0369 (0.0077–0.1523)	0.0507 (0.0031–0.1941)	0.59
IL-6 median (range)	0.0130 (0.0069–0.04000)	0.0127 (0.0035–0.0271)	0.65
c-REL median (range)	0.0611 (0.0301–0.1107)	0.0412 (0.02936–0.0554)	**0.006**
FOXP3 median (range)	0.0034 (0.0015–0.0082)	0.0027 (0.0008–0.0048)	0.15
T-BET median (range)	0.0064 (0.0016–0.0157)	0.0029 (0.0002–0.0078)	**0.017**
GATA3 median (range)	0.0057 (0.0039–0.0093)	0.0061 (0.0033–0.0178)	0.91
STAT3 median (range)	0.0299 (0.0131–0.1026)	0.0244 (0.0035–0.0474)	0.56
CXCR3 median (range)	0.0063 (0.0029–0.0137)	0.0044 (0.0019–0.0073)	0.08
c-MAF median (range)	0.0003 (0.0001–0.0009)	0.0002 (0.00002–0.0007)	0.06
AHR median (range)	0.0047 (0.0015–0.0167)	0.0060 (0.0033–0.01365)	0.59
CCR6 median (range)	0.0367 (0.0066–0.1539)	0.0387 (0.0013–0.1044)	0.99

P values indicating significant differences are shown in **bold.**

Table 22. Relationship between expression $CD4^+$ subset transcriptional regulators, chemokine receptors and inflammatory markers in $CD4^+$ T cells

	TNF	*PPARγ*	*PON2*	*CCR4*	*IL-6*	*c-REL*	*FOXP3*	*T-BET*	*GATA3*	*STAT3*	*CXCR3*	*c-MAF*	*AHR*	*CCR6*
TNF	–	**0.02**	**0.007**	**0.02**	0.23	0.39	**0.01**	0.13	**0.003**	0.20	0.21	0.98	**0.009**	0.28
PPARγ	**0.02**	–	**$5.5x10^{-6}$**	**$4.9x10^{-5}$**	**0.004**	0.07	**0.001**	**0.03**	**$2.1x10^{-4}$**	**0.006**	**0.04**	0.41	**0.03**	**0.02**
PON2	**0.007**	**$5.5x10^{-6}$**	–	**$1.1x10^{-4}$**	**0.01**	0.09	**$3.4x10^{-4}$**	**0.03**	**$3.5x10^{-4}$**	**0.02**	**0.03**	0.41	**0.02**	**0.03**
CCR4	**0.02**	**$4.9 x10^{-4}$**	**$1.1x10^{-4}$**	–	**0.001**	**0.04**	**0.002**	**0.04**	**0.001**	**0.001**	**0.01**	0.36	**0.04**	**0.003**
IL-6	0.23	**0.004**	**0.01**	**0.001**	–	**0.02**	**0.02**	0.12	**0.02**	**$4.9x10^{-5}$**	0.07	0.29	0.23	**0.001**
c-REL	0.39	0.07	0.09	**0.04**	**0.02**	–	0.08	0.79	0.08	0.06	0.08	0.31	0.89	0.14
FOXP3	**0.01**	**0.001**	**$3.4x10^{-4}$**	**0.002**	**0.02**	0.08	–	**0.03**	**$2.1x10^{-4}$**	**0.04**	0.09	0.55	**0.04**	0.11
T-BET	0.13	**0.03**	**0.03**	**0.04**	0.12	0.79	**0.03**	–	0.08	0.09	0.52	0.25	0.08	0.11
GATA3	**0.003**	**$2.1x10^{-4}$**	**$3.5x10^{-4}$**	**0.001**	**0.02**	0.08	**$2.1x10^{-4}$**	0.08	–	**0.03**	0.08	0.89	**0.01**	0.07
STAT3	0.20	**0.006**	**0.02**	**0.001**	**$4.9x10^{-5}$**	0.06	**0.04**	0.09	**0.03**	–	**0.04**	0.34	0.17	**$2.1x10^{-5}$**
CXCR3	0.21	**0.04**	**0.03**	**0.01**	0.07	0.08	0.09	0.52	0.08	**0.04**	–	0.42	0.11	**0.02**
c-MAF	0.98	0.41	0.41	0.36	0.29	0.31	0.55	0.25	0.89	0.34	0.42	–	0.78	0.29
AHR	**0.009**	**0.03**	**0.02**	**0.04**	0.23	0.89	**0.04**	0.08	**0.01**	0.17	0.11	0.78	–	0.14
CCR6	0.28	**0.02**	**0.03**	**0.003**	**0.001**	0.14	0.11	0.11	0.07	**$2.1x10^{-5}$**	**0.02**	0.29	0.14	–

All figures displayed are *P* values indicating the significance of the relationship between genes. *P* values indicating significant differences are shown in **bold**

6.4 Discussion

We have shown for the first time that *c-REL* (required for nTreg differentiation) and *T-BET* (required for Th1 differentiation) gene expression is decreased while TNF gene expression is increased in peripheral blood $CD4^+$ T cells in CF. While a Th2 and Th17 bias in CF has been widely reported, few studies have investigated expression of the key transcriptional regulators of $CD4^+$ T cell subsets, and many studies involving immune cells and their markers have focussed on the lungs rather than the periphery.

The transcription factor c-REL is known to control the differentiation of natural Tregs (nTreg) but is not essential for the development of iTregs (189). Therefore, decreased *c-REL* expression could suggest that nTregs are decreased in CF, but because we did not see reduced Treg levels in our previous flow cytometric analyses where nTregs and iTreg were measured as one population, a compensatory effect could be occurring whereby there are increases in peripherally induced iTregs. Secondly, *c-REL* is also known to be essential for the generation of IL-2, IL-3, IFNγ and GMCSF by T cells (349). Production of both IFNγ and GMCSF has been shown to be decreased in CF lungs and peripheral blood (262, 301) and to be correlated with worse outcomes in response to common CF pathogens such as *P. aeruginosa*. Reduced *c-REL* expression by $CD4^+$ T cells could therefore be an underlying factor in the Th2 bias seen in CF (51, 53). Indeed, a previous study has shown that *c-REL* promotes Th1 and Th17 responses during infection with the pathogen *Leishmania major* (350).

In addition to a decrease in *c-REL* expression, we also saw a decrease in expression of *T-BET*, the primary transcriptional regulator of the Th1 lineage, and a trend towards decreased expression of *CXCR3*, a Th1 specific marker. These decreases again could be contributing factors in the Th2 dominated immune response identified in CF, as seen in the lung (216, 262, 267) and periphery by us.

Interestingly, we saw increased levels of *TNF* expression in $CD4^+$ cells from CF patients. Increases in the levels of TNF protein, an inflammatory cytokine, in CF have been reported extensively, particularly in the lung and plasma (256, 351-355). However, there have been no studies investigating the contribution of $CD4^+$ T cells to TNF production. However, Mueller and colleagues have shown that *TNF* expression is increased in CF lymphocytes as a result of the increased nuclear localisation of NFAT caused by mutated *CFTR* (22), which is consistent with our findings. Increased *TNF* expression may contribute to the hyperinflammatory environment characteristic of CF.

When considering the relationships between the various immune cell markers it is apparent that there is an overarching regulation, because expression of many of the markers is correlated. Regulation of only *c-MAF* (required for Tr1 differentiation) and *c-REL* (required for nTreg differentiation) appears independent of the other markers measured. The strong relationships between $CD4^+$ transcriptional regulators and inflammatory markers highlights the extent to which changes in the differentiation of one $CD4^+$ subset can affect others. It also demonstrates that any action to correct individual dysregulated subsets could have many off-target effects and suggests that correction of CFTR function may correct much of the immune dysfunction seen in people with CF.

One strength of this study is the investigation of these markers in immune cells in the periphery, reducing direct bias caused by chronic infection in the lungs. However, while we have investigated the primary transcriptional regulators of $CD4^+$ subsets, there are many others that play a role in subset differentiation. Also, given the relatively small numbers in the CF group, analysis of the relationships between clinical variables and expression of markers resulted in quite small subgroups in which some relationships may not have been detectable.

In summary, we have shown decreases in expression of the transcription factors *c-REL* (required for nTreg differentiation) and *T-BET* (required for Th1 differentiation) in $CD4^+$ T cells in CF, both of which are involved in the generation of Th1 cells or Th1-related cytokines. Peripheral $CD4^+$ T cells were also shown to express increased levels of mRNA for the inflammatory cytokine TNF. These changes in gene expression could be contributing factors in the dysregulation of the immune response in CF.

CHAPTER 7

Flow Cytometric and RT-qPCR Analysis of Peripheral Blood Immune Cells in Carriers of a Mutated Cystic Fibrosis Transmembrane Conductance Regulator Gene

7.1 Introduction

Lung damage is the primary cause of morbidity and mortality in CF with inappropriate immune responses believed to play a contributing role. We have previously shown changes in both innate and adaptive immune cells such as $CD4^+$ T cells, monocytes, DCs, NK cells and MDSCs in the peripheral blood of people with CF. However, the role of CFTR in this immune dysfunction has yet to be fully elucidated.

There is limited research, particularly in primary human cells, investigating the direct effects of dysfunctional *CFTR* on the immune response. The presence of inflammation prior to any detectable infection has been shown in children with CF (30), with current evidence suggesting that CFTR plays a role in neutrophil, macrophage and T cell function. Studies in human neutrophils have indicated that CFTR affects microbicidal activity in these cells by way of reduced chloride anions that in turn reduces the ability of the phagosome to produce hypochlorous acid, a critical component in bacterial killing (96-98), while studies in mice have shown a CFTR-mediated activation of NF-κB that promotes secretion of proinflammatory cytokines (356, 357). Dysfunctional CFTR in human macrophages has also been reported to result in reduced microbicidal activity (259) while studies in mouse models have indicated that dysfunctional CFTR results directly in increased production of proinflammatory cytokines (257, 358, 359). Only one study, using a mouse model, has investigated the role of dysfunctional CFTR in T cells. This study demonstrated that CFTR directly affects T cell responses, resulting in aberrant cytokine production (22).

To clarify whether the changes that we have seen in the peripheral immune cells in people with CF are mediated by infection and inflammation or by an inherent defect associated with *CFTR* mutations, we investigated these cells in *CFTR* heterozygotes. *CFTR* heterozygotes, or CF carriers, are those with one normal and one defective *CFTR* allele, and appear clinically normal, although there is evidence to suggest that *CFTR* carriers are more prone to bronchiectasis (360), inflammatory illnesses such as asthma (361) and chronic rhinosinusitis (362) and to other conditions including idiopathic chronic pancreatitis (363) and congenital bilateral absence of the vas deferens (364). These studies provide further evidence to suggest that the immune abnormalities seen in CF patients may, at least in part, be mediated by *CFTR* mutations rather than by chronic infection and inflammation.

To evaluate the role of *CFTR*-mediated immune dysfunction in CF we firstly investigated the proportions of $CD4^+$ effector and regulatory T cell subsets, monocytes, DCs, NK cells and

MDSCs in *CFTR* carrier PBMC and the expression of the Th1-, Th2- and Th17-associated chemokine receptors CXCR3, CCR4 and CCR6 using flow cytometry. Secondly, using RT–qPCR, we examined the relative mRNA expression of $CD4^+$ subset transcriptional regulators, chemokine receptors and inflammatory markers in $CD4^+$ T cells. The results for all these parameters in *CFTR* carrier PBMC were compared with those obtained for healthy controls and people with CF.

7.2 Materials and Methods

The same analyses described in Chapters 3–5 for CF patients and controls were undertaken using PBMC from *CFTR* carriers. Peripheral blood samples were obtained and PBMC isolated as described in Chapter 2. PBMC were analysed to determine proportions of $CD4^+$ effector (Th1, Th2, Th17) and regulatory (Treg, Tr1 and Th3) subsets and the expression of CXCR3, CCR4 and CCR6 chemokine receptors, as described in Chapters 3 and 4, respectively. PBMC were then analysed to determine proportions of monocytes, dendritic cells, natural killer cells and myeloid derived suppressor cells, as described in Chapter 5. Lastly $CD4^+$ T cells were isolated and analysed for relative mRNA expression of CD4 transcriptional regulators, chemokine receptors and inflammatory markers, as described in Chapter 6.

These analyses included only adult CF carriers (22–47 years, n = 9–13), adult controls (22–50 years, n = 15–51) and adult CF patients (19–54 years, n = 10–19). Participant characteristics are detailed in appendices 2.1 and 2.2. Of note, 48% of the carrier population carried a DF508 *CFTR* mutation.

7.3 Results

7.3.0 Comparison of CF Carriers with Healthy Control and CF Groups

Flow cytometric and gene expression analyses of CF carrier PBMC revealed that in many cases this group appeared similar to CF patients or intermediate between CF patients and controls, rather than to healthy controls. Only data for the subsets of interest that differ between the three groups are described below, but all subset data from flow cytometric analyses of CF carrier PBMC are detailed in Appendix 3. Of note, there were no significant differences between the median age and sex distributions of the control, CF carrier and CF groups.

7.3.1 CD4 Effector and Regulatory Subset Analysis

Analysis of $CD4^+$% and $CD4^+$ effector (Th1, Th2, Th17) and regulatory ($FOXP3^+$ Treg, IL-10^+ Tr1, $TGF\beta^+$ Th3) populations revealed that CF carriers had a higher $CD4^+$% compared with people with CF ($P = 0.04$) (Fig. 14) and a trend toward higher $CD4^+$% compared with healthy controls ($P = 0.051$) (Fig. 14). Proportions of $CD4^+$ effector subsets were unchanged in CF carriers compared with both CF and healthy controls (Fig. 14). Proportions of the regulatory $FOXP3^+$ Tregs and IL-10^+ Tr1 cells were both increased in CF carriers compared with healthy controls ($P = 0.0004$ and $P = 0.001$ respectively) but only $FOXP3^+$ Tregs were increased compared with CF ($P = 0.03$) (Fig. 15), although IL-10^+ Tr1 cells were trending toward being higher in CF carriers compared with CF. No difference was seen in the proportion of $TGF\beta^+$ Th3 cells between CF carriers and healthy controls although there was a trend towards a decrease in CF carriers compared with CF patients ($P = 0.08$) (Fig. 15). However, it must be noted that in this analysis of CD4 effector and regulatory subsets, the median age of the CF carriers was significantly greater than that of either CF patients or healthy controls ($P = 0.018$ and $P = 0.002$ respectively).

7.3.2 $CD4^+$ and Treg Homing Marker Analysis

Analysis of memory $CD4^+$ cells expressing the Th1-, Th2- and Th17-associated homing markers CXCR3, CCR4 and CCR6 respectively, revealed no changes in the levels of $CD4^+$, memory $CD4^+$, $CXCR3^+CD4^+$ or $CCR4^+CD4^+$ cells in CF carriers compared with either CF patients or healthy controls (Fig. 16). However, a significant decrease in $CCR6^+CD4^+$ cells ($P = 0.026$) was seen in CF carriers compared with healthy controls (Fig. 16).

Examination of Treg cells and their expression of these homing markers revealed a normal overall level of Treg cells in CF carriers compared with healthy controls but a decrease in memory Tregs (P = 0.026) (Fig. 17). CF carriers also had lower levels of $CCR4^{+}$Tregs (Th2-associated, P = 0.039) but normal levels of $CXCR3^{+}$ Tregs compared with healthy controls (Fig. 17). Interestingly, while memory $CCR6^{+}$Tregs were decreased in CF carriers compared with healthy controls (P = 0.023), naïve $CCR6^{+}$ Tregs were increased compared with the CF group (P = 0.036) (Fig. 18).

7.3.3 Innate Immune Cell Analysis

All innate immune cell subsets that were measured in CF patients and healthy controls (described in Table 15) were assessed in CF carriers but those that did not differ between any of the three groups are not discussed below and are detailed in Appendix 3.3. $CD14^{+}$ and $CD14^{-}$ MDSCs were similar in CF carriers compared with both healthy controls and CF patients (Fig. 19). DCs showed a trend towards being higher in carriers than in healthy controls (P = 0.07) but did not differ significantly from the CF group (Fig. 20). Monocytes analysed as a single heterogeneous population were increased in CF carriers compared with controls (P = 0.04) but again did not differ from the CF group (Fig. 21). For the individual monocyte subsets categorised based on CD14 and CD16 expression, 'classical' monocytes trended towards being higher in CF carriers compared with healthy controls (P = 0.06) while 'non-classical' monocytes were unchanged; neither population differed significantly from the CF group (Fig. 21). Lastly, NK cells assessed as one heterogeneous population based on NKP46 and CD16 expression were lower in CF carriers than in healthy controls (P = 0.001) but did not differ from the CF group (Fig. 22). When NK cells were divided into subpopulations based on CD16 and CD56 expression, $CD16^{hi}CD56^{int}$ (cytotoxic) NKs were lower (P = 0.049) while $CD16^{lo/neg}CD56^{hi}$ (cytokine-producing) NKs were higher (P = 0.008) in CF carriers compared with healthy controls but neither population differed between CF carriers and CF patients (Fig. 22).

7.3.4 Multiexperiment Viewer (MeV) Bioinformatic Analysis of Peripheral Innate Immune Cell Subsets

Multiexperiment Viewer (MeV) was used in order to visualise common trends/patterns between the immune profiles of CF patients, carriers and healthy controls. Firstly, all immune cell subsets detailed in Fig.19-22 were analysed in all CF patients, carriers and healthy controls. A heat map was generated that included only the subsets that differed significantly

between controls and CF patients, to allow visualisation of patterns of upregulation/downregulation, comparative to the median, between the participant groups. (Fig.23). As previously shown (see section 5.3.3) there was a trend towards separate clustering of CF patients and controls while the CF carriers showed no distinct clustering pattern and were scattered amongst both the CF and control groups.

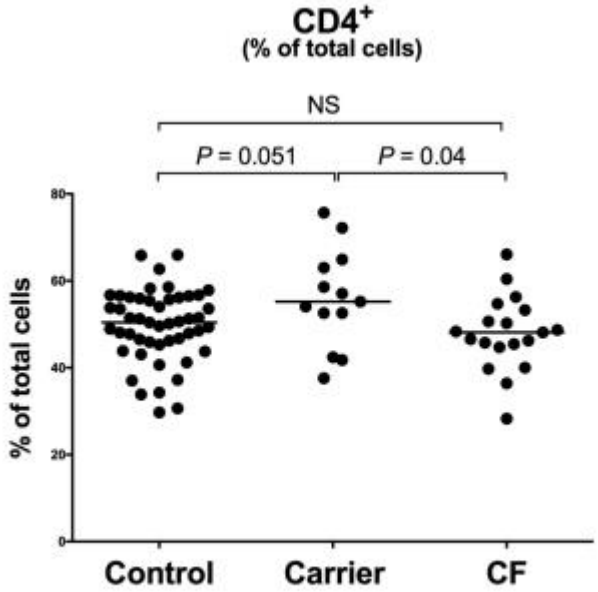
Next, in order to further elucidate how the immune cell profile of CF carriers was related to healthy controls, a heat map was generated to visualise the significantly different subsets (Fig.24). CF carriers tended to cluster separately from healthy controls with a few exceptions. A heat map of the significant differences between CF carriers and CF patients was unable to be generated as there were no significant differences in innate immune cell subsets between these two groups.

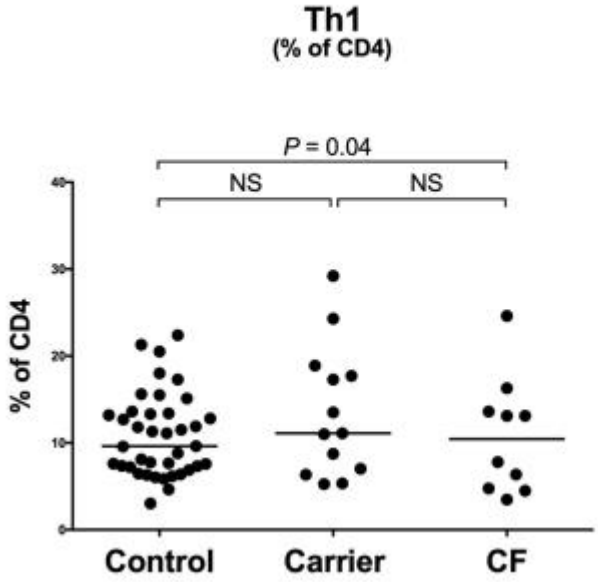
7.3.5 $CD4^+$ T Cell Gene Expression Analysis

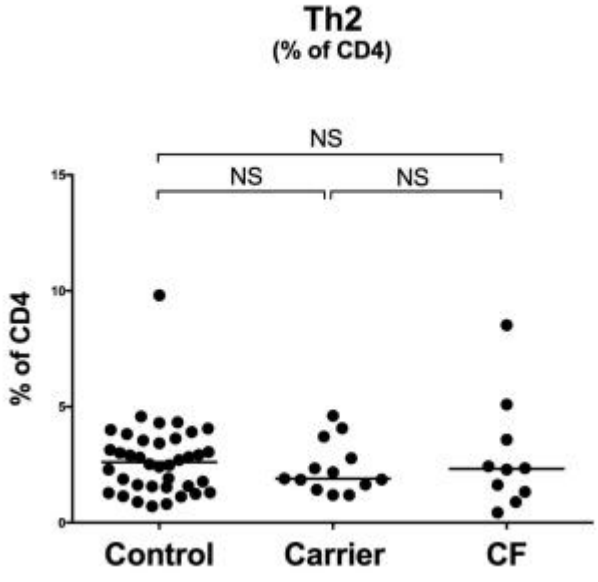
Gene expression analysis of CD4 transcriptional regulators (Table 23) revealed that CF carriers differed from healthy controls by having lower expression of *c-REL* (required for nTreg differentiation) and *c-MAF* (required for Tr1 differentiation) and higher expression of AHR (required for Tr1 differentiation). CF carriers also differed from CF patients, with higher expression of *T-BET* (required for Th1 differentiation) and AHR (required for Tr1 differentiation). No differences were seen between any group for the expression of FOXP3 and STAT3 or the chemokine receptors CXCR3, CCR4 and CCR6.

Investigation of expression of the inflammatory cytokines IL-6 and TNF in CF carriers showed that IL-6 was lower and TNF expression was higher than in healthy controls but neither differed from that in CF patients.

Expression of PPARγ and PON2, which have previously been shown to be correlated in CF BAL cells, were found not to differ significantly between any of the groups.


CD4+
(% of total cells)
NS
P = 0.051
P = 0.04
% of total cells
0
20
40
60
80
Control
Carrier
CF


Th1
(% of CD4)
P = 0.04
NS
NS
% of CD4
0
10
20
30
40
Control
Carrier
CF


Th2
(% of CD4)
NS
NS
NS
% of CD4
0
5
10
15
Control
Carrier
CF

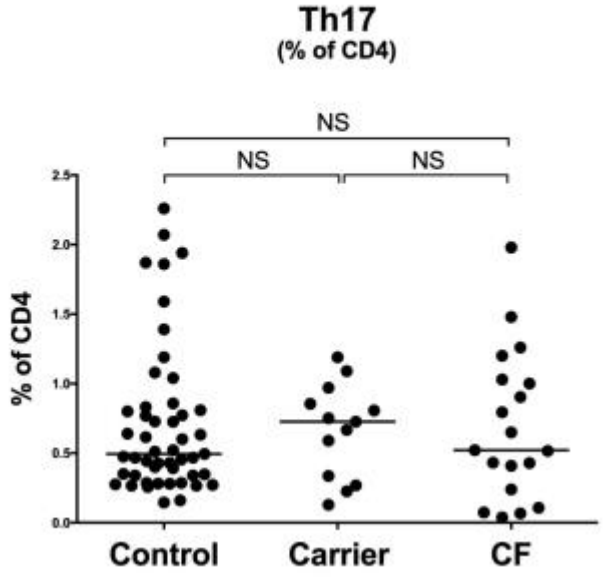

Th17
(% of CD4)
NS
NS
NS
% of CD4
0.0
0.5
1.0
1.5
2.0
2.5
Control
Carrier
CF

Fig. 14. $CD4^+$ effector T cell subset percentages in CF carriers compared with healthy control and CF group. Proportions of $CD4^+$, Th1, Th2 and Th17 cells are shown. $CD4^+$ cells were measured as a percent of total cells while Th1 ($CD4^+IFN\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$) and Th17 ($CD4^+IL\text{-}17^+$) cells were measured as a percent of $CD4^+$ cells. The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.

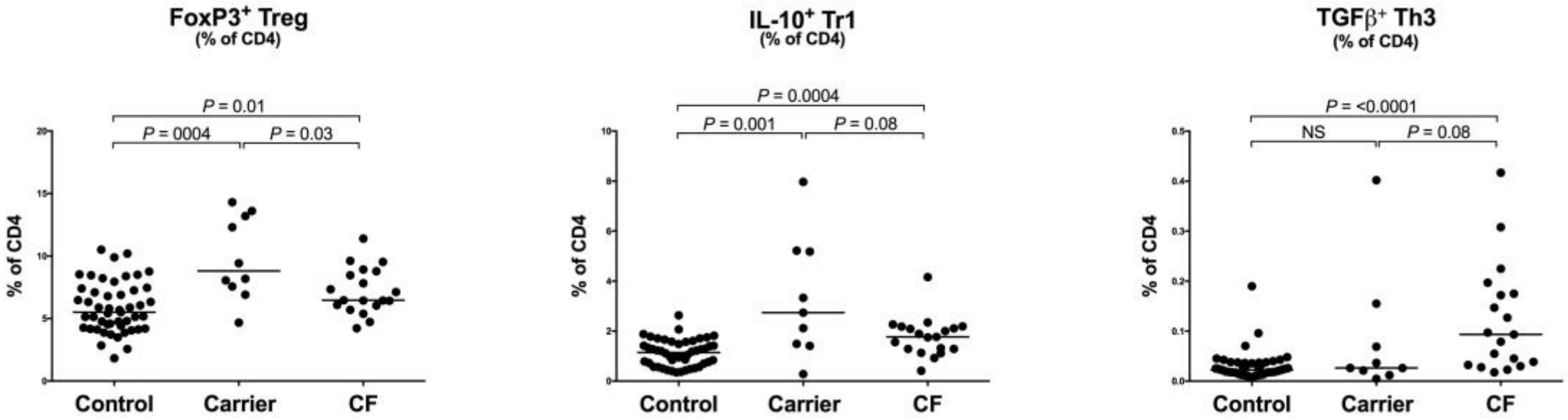
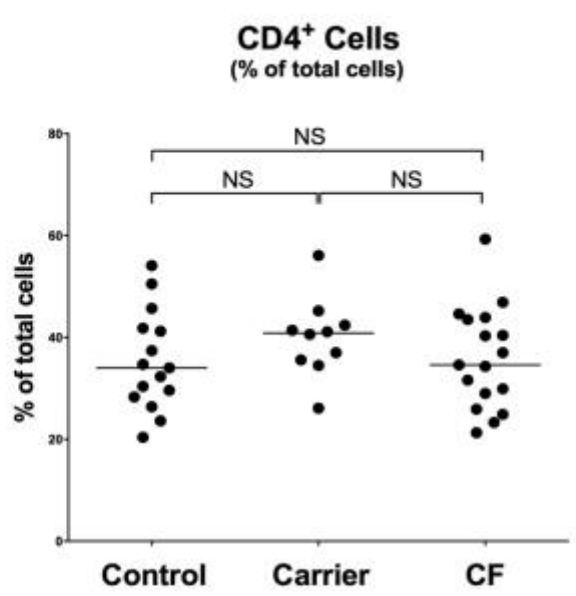
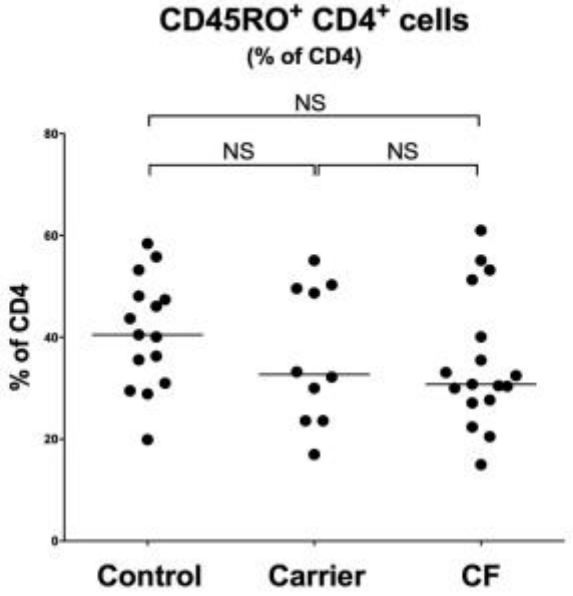
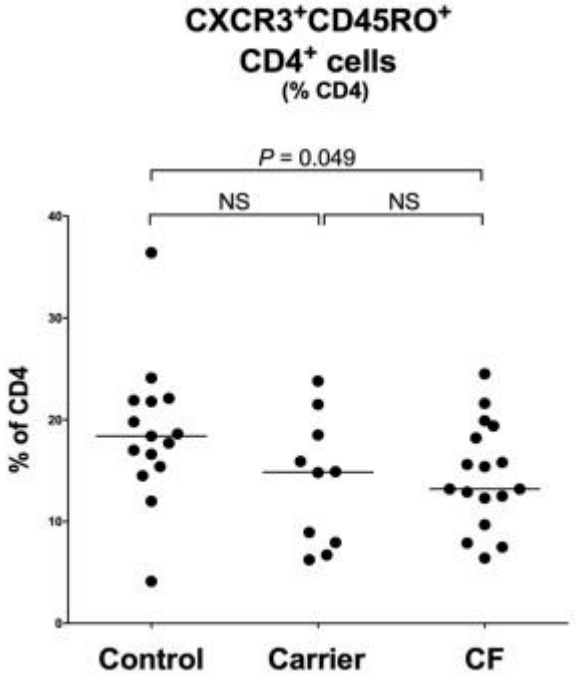
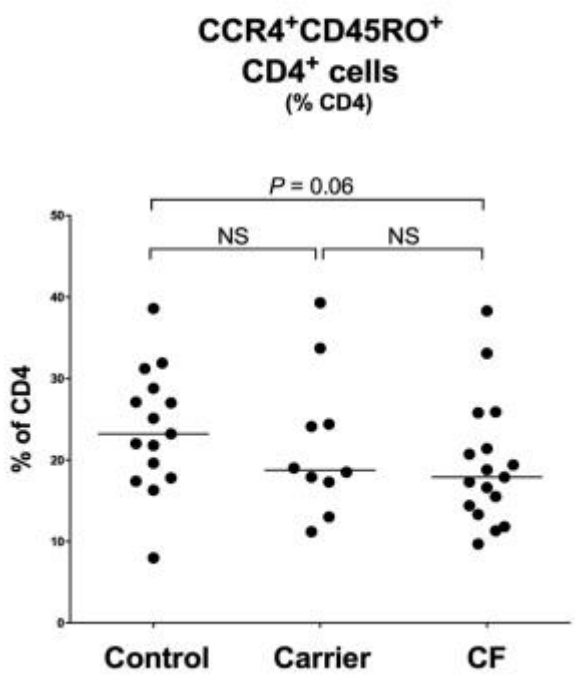


Fig. 15. CD4$^+$ regulatory T cell subset percentages in CF carriers compared with healthy control and CF group. Proportions of FOXP3$^+$ Treg (CD4$^+$FoxP3$^+$), IL-10$^+$ Tr1 (CD4$^+$IL-10$^+$), and TGFβ$^+$ Th3 (CD4$^+$ TGFβ$^+$) cells are shown. All populations are measured as a percent of CD4$^+$ cells. The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.


CD4+ Cells
(% of total cells)
% of total cells
NS
NS
NS
Control
Carrier
CF


CD45RO+ CD4+ cells
(% of CD4)
% of CD4
NS
NS
NS
Control
Carrier
CF


CXCR3+CD45RO+
CD4+ cells
(% CD4)
% of CD4
P = 0.049
NS
NS
Control
Carrier
CF


CCR4+CD45RO+
CD4+ cells
(% CD4)
% of CD4
P = 0.06
NS
NS
Control
Carrier
CF

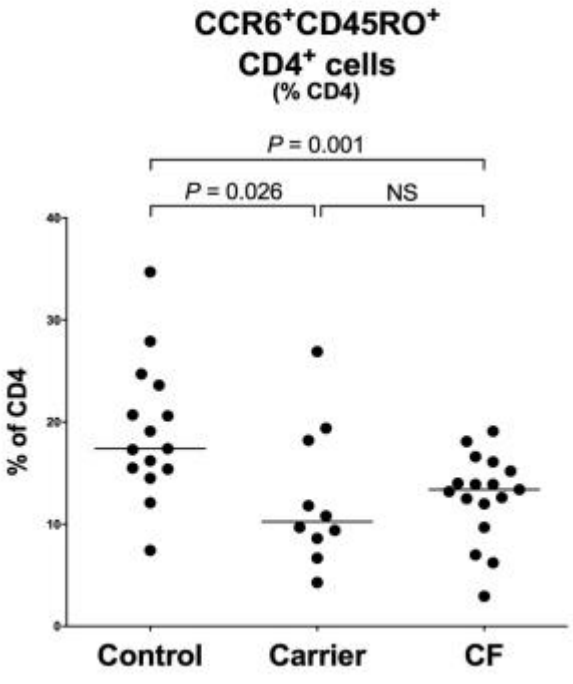
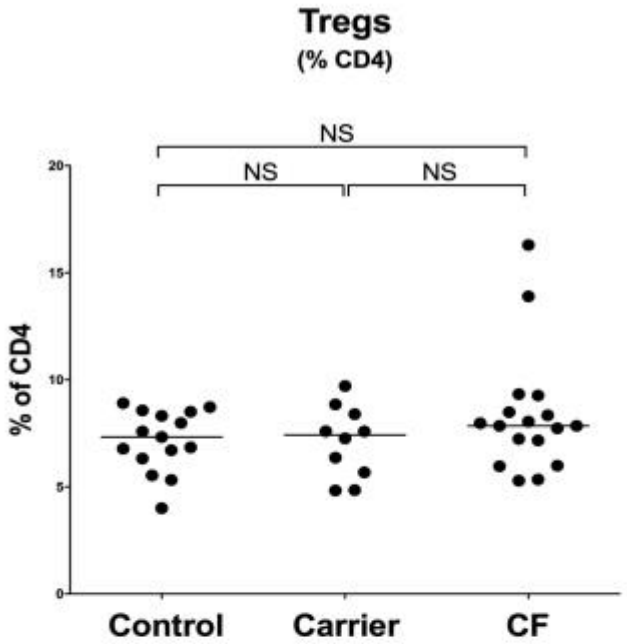
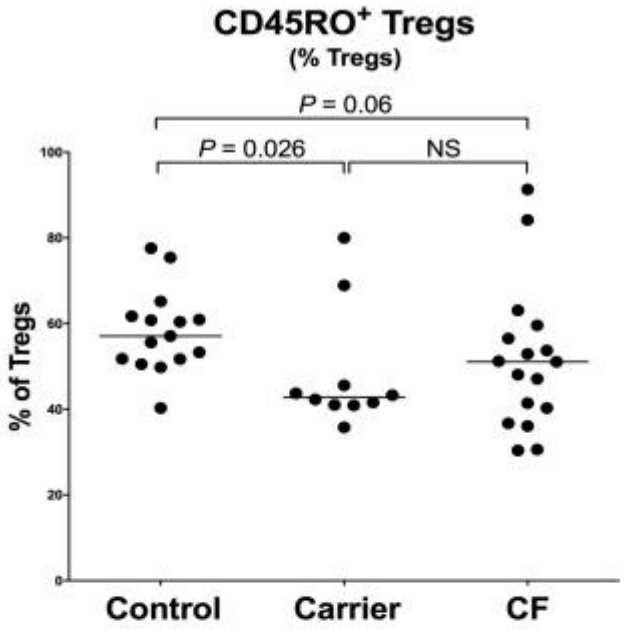
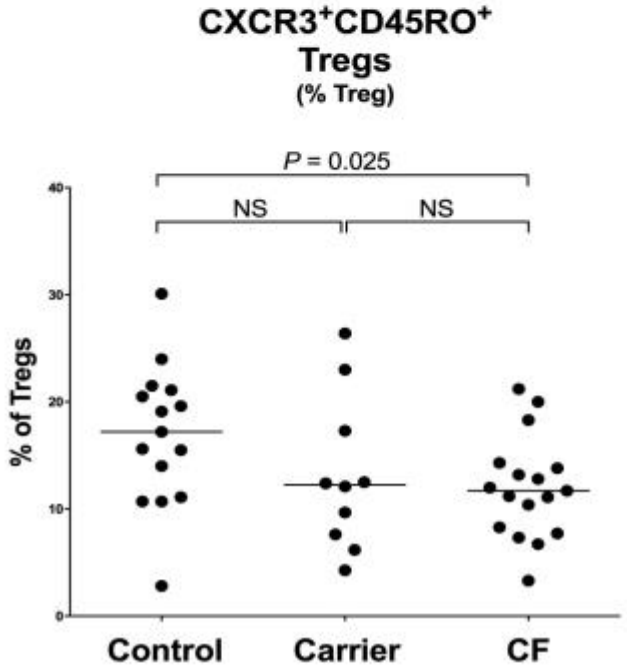

CCR6+CD45RO+
CD4+ cells
(% CD4)
% of CD4
P = 0.001
P = 0.026
NS
Control
Carrier
CF

Fig. 16. Percentages of CD4+ (total and memory), CCR4$^+$, CCR6$^+$ and CXCR3$^+$ CD4$^+$ T cells in CF carriers compared with healthy control and CF group. Proportions of CD4$^+$, CD45RO$^+$CD4$^+$, CXCR3$^+$CD45RO$^+$CD4$^+$, CCR4$^+$CD45RO$^+$CD4$^+$ and CCR6$^+$CD45RO$^+$CD4$^+$ cells are shown. CD4$^+$ cells were measured as a percent of total live cells while CD45RO$^+$CD4$^+$, CXCR3$^+$CD45RO$^+$CD4$^+$, CCR4$^+$CD45RO$^+$CD4$^+$ and CCR6$^+$CD45RO$^+$CD4$^+$ cells were all measured as a percent of CD4$^+$ cells. The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.


Tregs
(% CD4)
NS
NS
NS
% of CD4
20
15
10
5
0
Control
Carrier
CF


CD45RO+ Tregs
(% Tregs)
P = 0.06
P = 0.026
NS
% of Tregs
100
80
60
40
20
0
Control
Carrier
CF


CXCR3+CD45RO+
Tregs
(% Treg)
P = 0.025
NS
NS
% of Tregs
40
30
20
10
0
Control
Carrier
CF

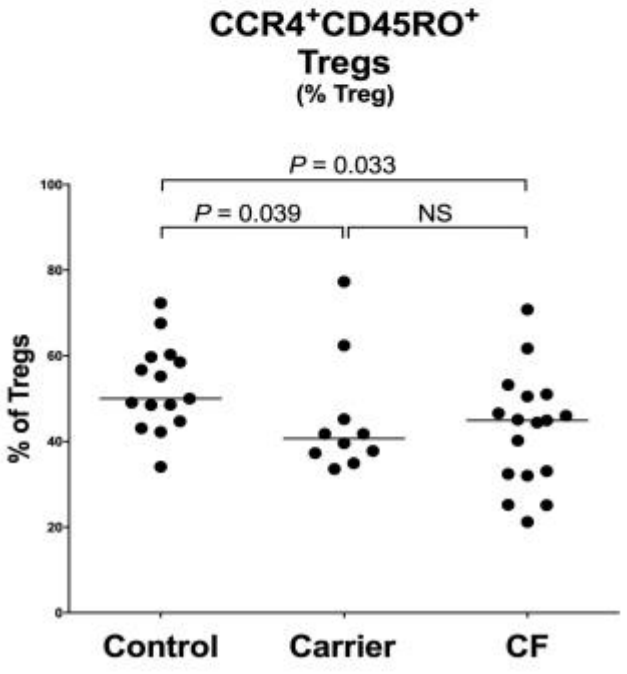

CCR4+CD45RO+
Tregs
(% Treg)
P = 0.033
P = 0.039
NS
% of Tregs
100
80
60
40
20
0
Control
Carrier
CF

Fig. 17. Percentages of Tregs (total and memory), CXCR3$^+$ and CCR4$^+$ memory Treg cells in CF carriers compared with healthy controls and the CF group. Proportions of Treg, CD45RO$^+$Treg, CXCR3$^+$CD45RO$^+$Treg and CCR4$^+$CD45RO$^+$Treg cells are shown. Treg cells were measured as a percent of CD4$^+$ cells while CD45RO$^+$Treg, CXCR3$^+$CD45RO$^+$Treg and CCR4$^+$CD45RO$^+$Treg cells were all measured as a percent of Treg cells. Tregs are defined as CD4$^+$CD25$^+$CD127^{lo}. The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.

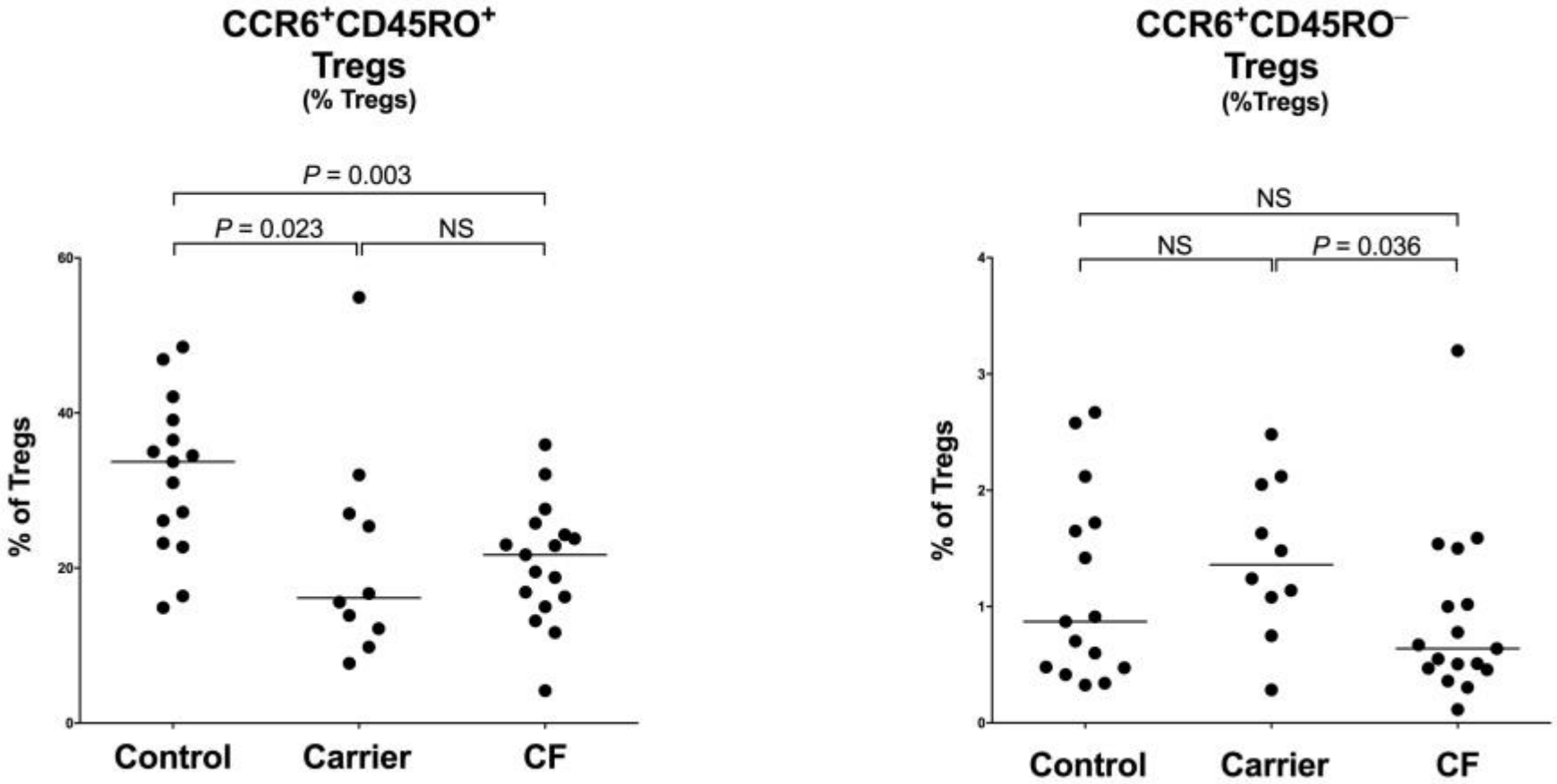


Fig. 18. Percentages of CCR6$^+$ memory and naïve Treg cells in CF carriers compared with healthy control and CF group. Proportions of CCR6$^+$CD45RO$^+$Treg and CCR6$^+$CD45RO$^-$Treg cells are shown. All populations were measured as a percent of Treg cells. Tregs are defined as CD4$^+$CD25$^+$CD127^{lo}. The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.

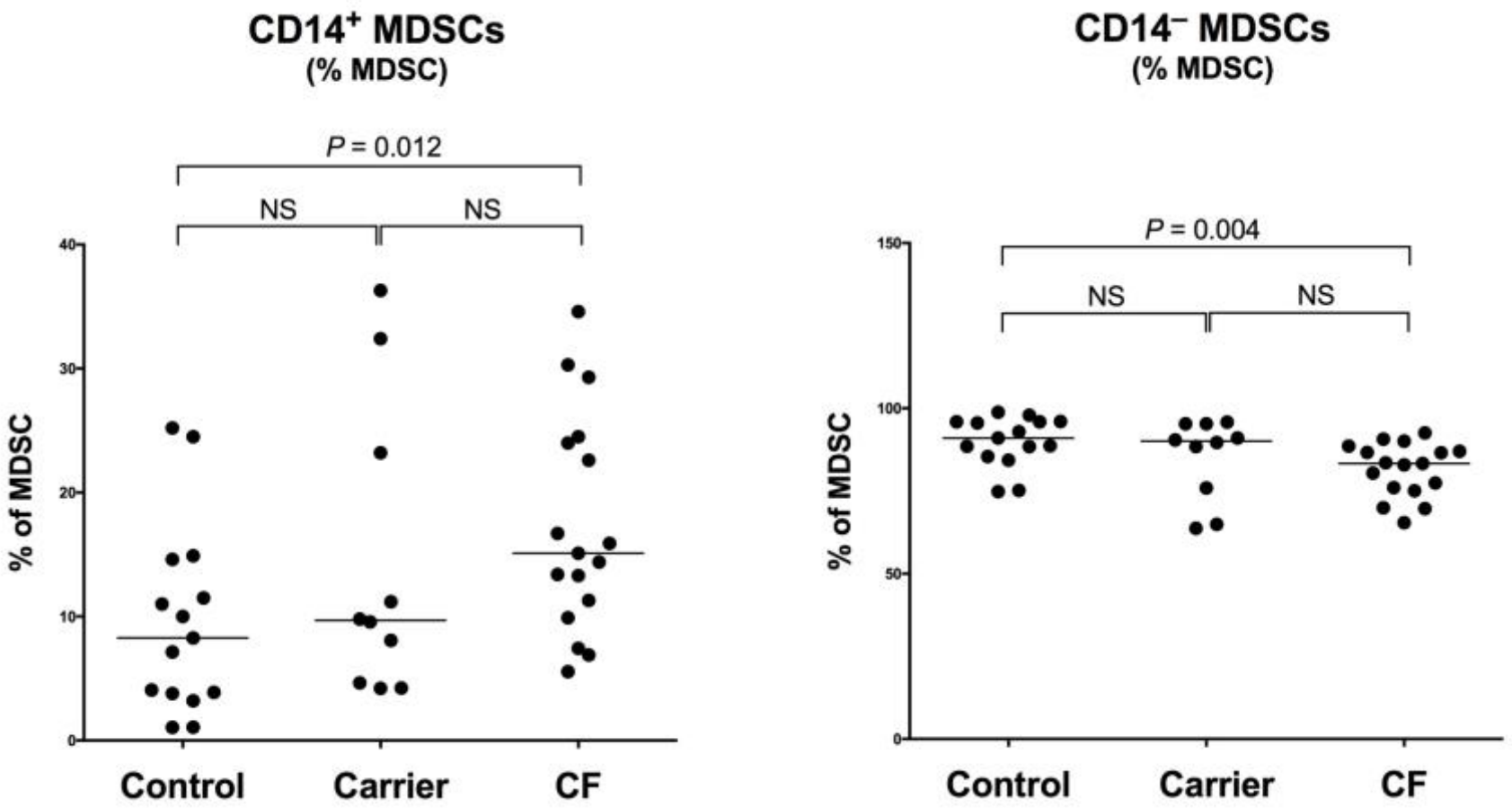


Fig. 19. Percentages of CD14$^{+/-}$ myeloid derived suppressor cells in CF carriers compared with healthy control and CF group. All populations are measured as a percent of total MDSCs (CD3^{-}CD56^{-}NKP46^{-}CX3CR1^{-}CD16^{+}CD11b^{+}). The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.

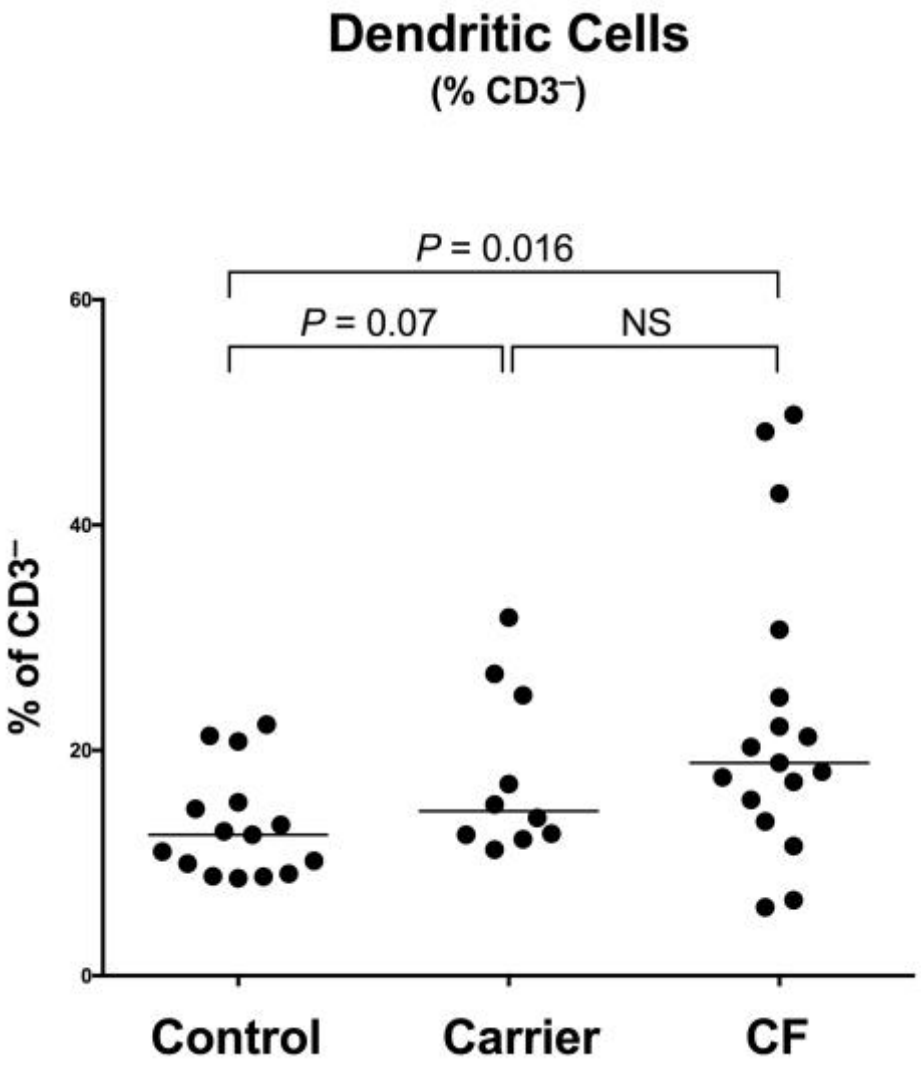
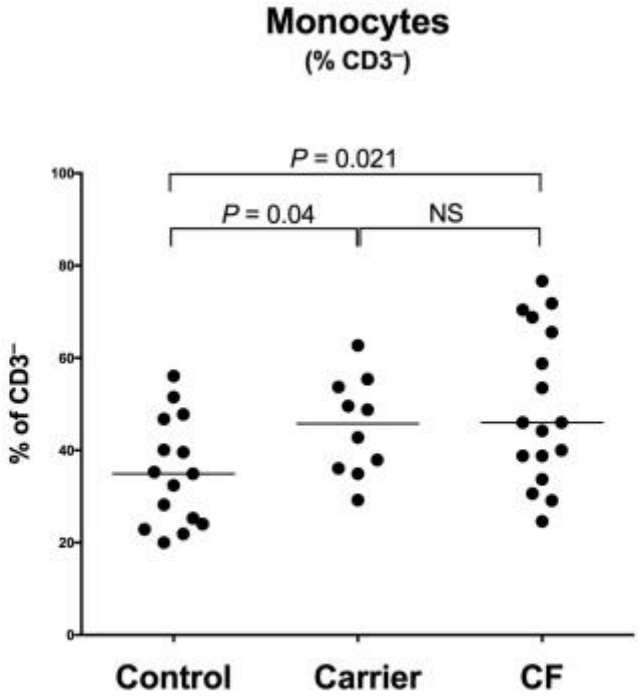
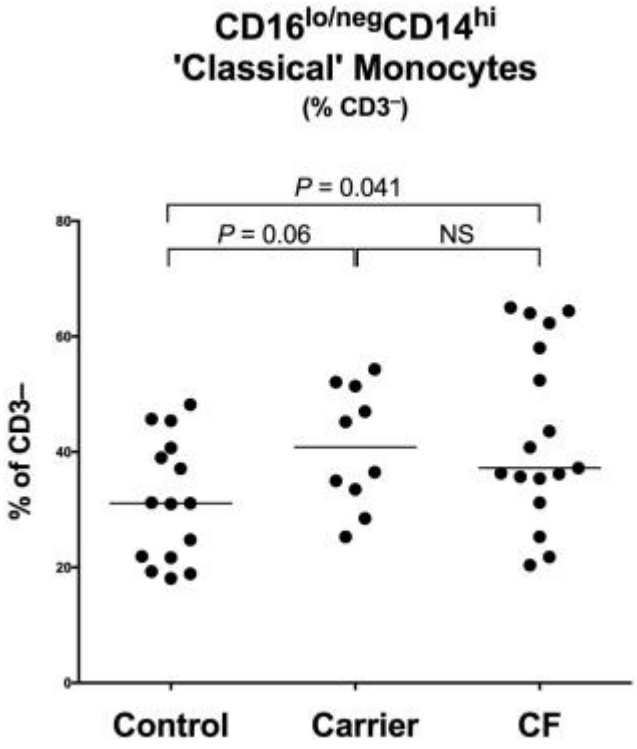


Fig. 20. Percentages of dendritic cells in CF carriers compared with healthy control and CF group. DC populations ($CD3^-CD56^-NKP46^-CD14^-CD16^-$) were measured as a percent of $CD3^-$ cells. The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.


Monocytes
(% CD3⁻)
P = 0.021
P = 0.04
NS
% of CD3⁻
100
80
60
40
20
0
Control
Carrier
CF


CD16lo/negCD14hi
'Classical' Monocytes
(% CD3⁻)
P = 0.041
P = 0.06
NS
% of CD3⁻
80
60
40
20
0
Control
Carrier
CF

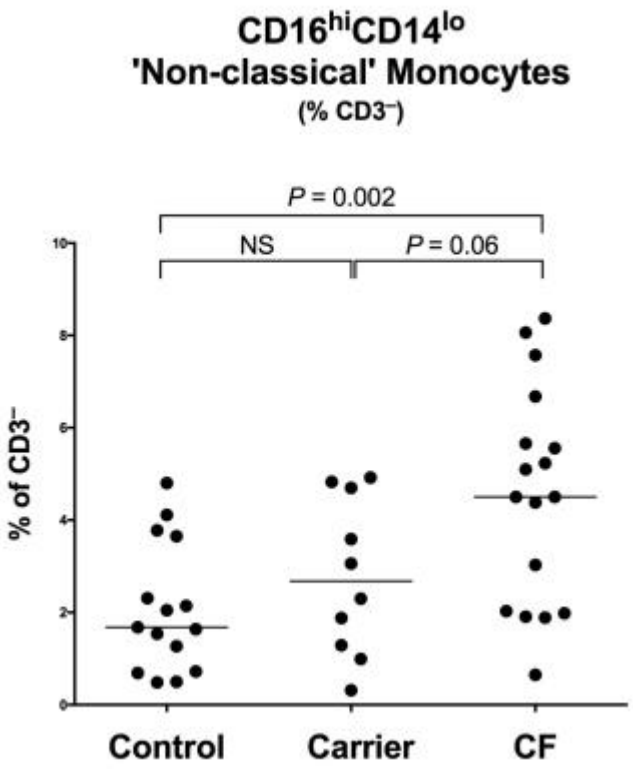
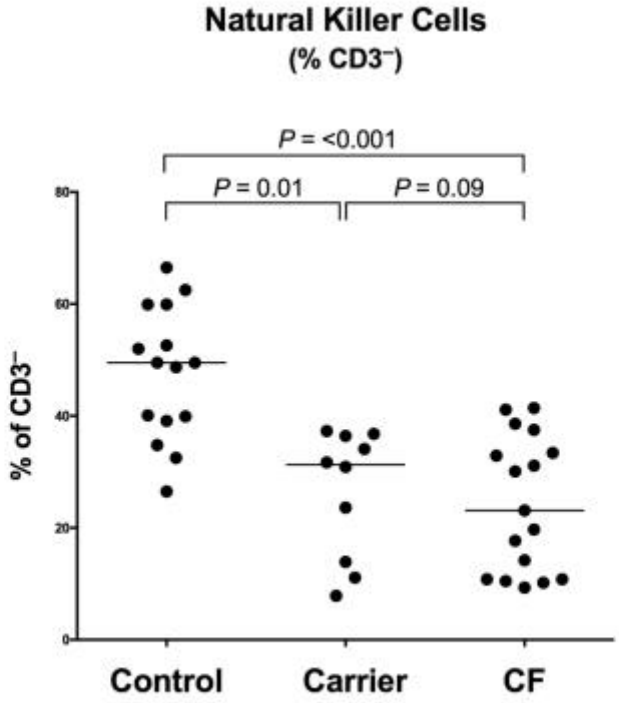
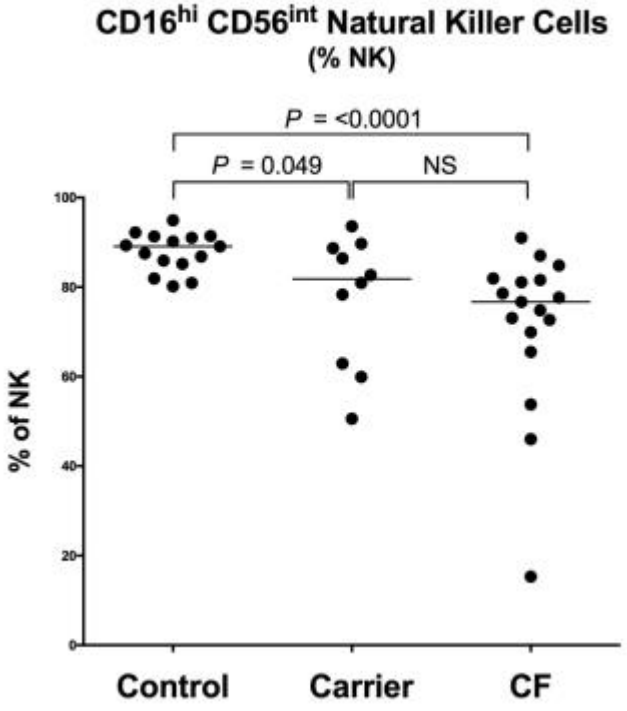

CD16hiCD14lo
'Non-classical' Monocytes
(% CD3⁻)
P = 0.002
NS
P = 0.06
% of CD3⁻
10
8
6
4
2
0
Control
Carrier
CF

Fig. 21. Percentages of monocytes and monocyte subsets in CF carriers compared with healthy control and CF group. Proportions of monocytes ($CD3^{-}CD56^{-}NKP46^{-}CX3CR1^{+}CD14^{lo/hi}CD16^{-/+}$) and of 'classical' $CD16^{lo/neg}CD14^{hi}$ and 'non-classical' $CD16^{hi}CD14^{lo}$ monocyte subsets are shown. Monocytes were measured as a percent of $CD3^{-}$ cells while 'classical' $CD16^{lo/neg}CD14^{hi}$ and 'non-classical' $CD16^{hi}CD14^{lo}$ monocyte subsets were measured as a percent of total monocytes. The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.


Natural Killer Cells
(% CD3⁻)
P = <0.001
P = 0.01
P = 0.09
% of CD3⁻
Control
Carrier
CF


CD16hi CD56int Natural Killer Cells
(% NK)
P = <0.0001
P = 0.049
NS
% of NK
Control
Carrier
CF

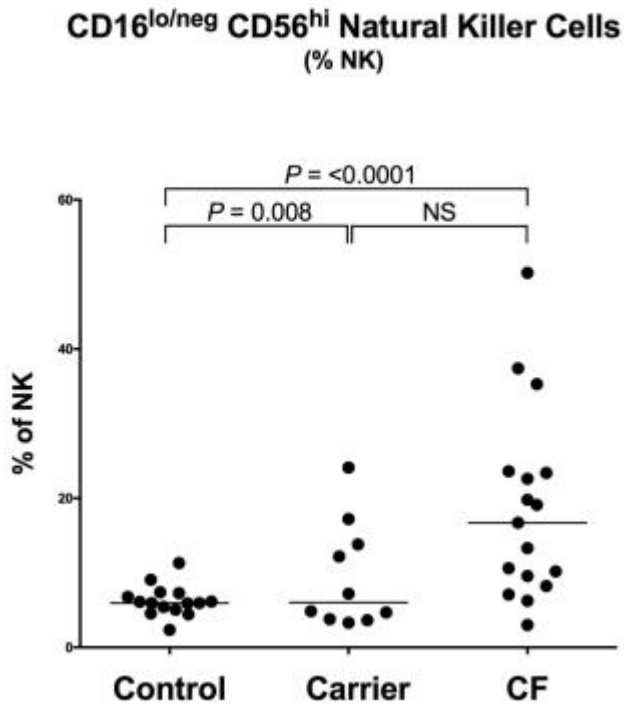
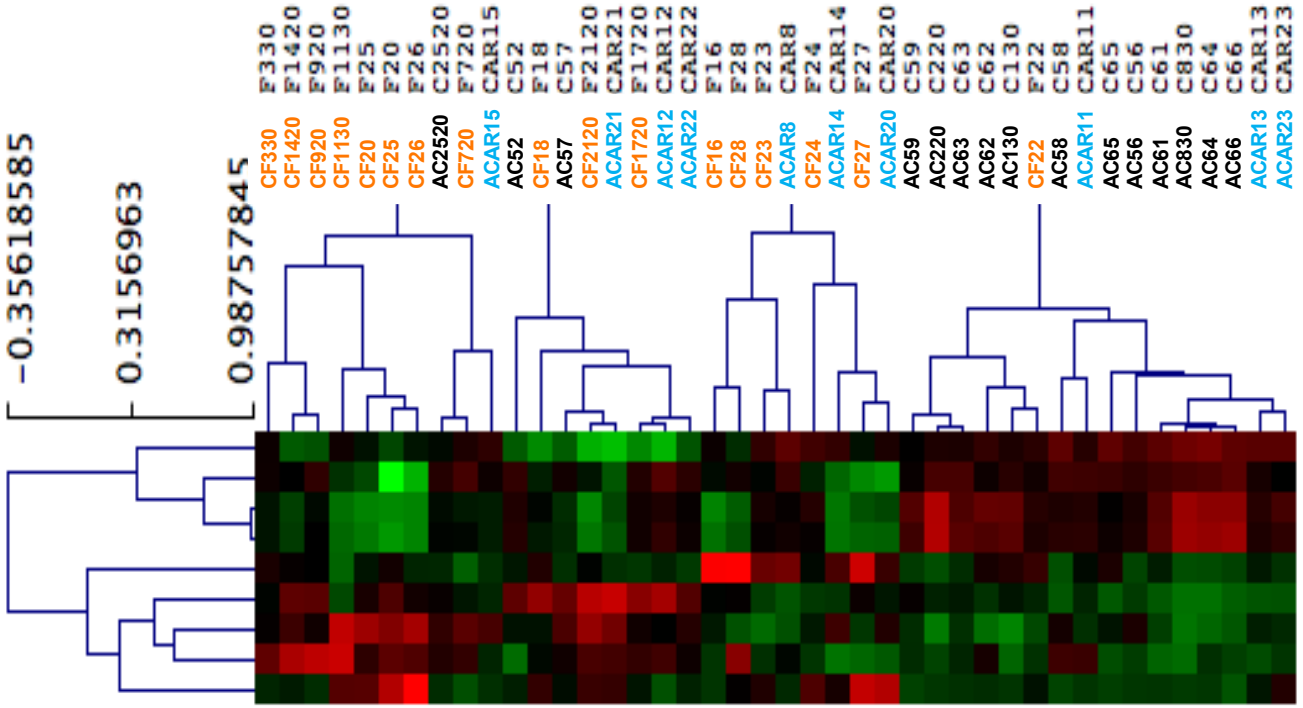

CD16lo/neg CD56hi Natural Killer Cells
(% NK)
P = <0.0001
P = 0.008
NS
% of NK
Control
Carrier
CF

Fig. 22. Percentages of natural killer and natural killer cell subsets in CF carriers compared with healthy control and CF group. Proportions of NK cells ($CD3^-$ $CD56^+NKP46^+$ $CD16^{-/+}$) and of $CD16^{hi}CD56^{int}$ and $CD16^{lo/neg}CD56^{hi}$ NK cell subsets are shown. NK cells were measured as a percent of $CD3^-$ cells while $CD16^{hi}CD56^{int}$ and $CD16^{lo/neg}CD56^{hi}$ NK cell subsets were measured as a percent of total NK cells. The significance of differences between populations was assessed using a Mann-Whitney *U* test. Horizontal bars indicate the median.



Controls

CF Carriers

CF

Fig. 23. Heat map of significantly different monocyte, natural killer, dendritic and myeloid derived suppressor cell subset percentages in CF patients, CF carriers and healthy controls. Raw data were imported in txt. file format to MeV. Data were normalised across rows and a nonparametric Kruskal–Wallis test was performed to determine differences in the proportions of each subset between groups. An unsupervised clustering analysis (hierarchical clustering) was performed using a nonparametric Spearman rank correlation distance metric and average linkage clustering. A heat map expression image was created to visualise results. Rows correspond to the immune cell subsets and columns correspond to individual subjects. Black squares indicate unchanged relative expression, green squares indicate down regulated relative expression and red squares indicate up regulated relative expression of that variable.

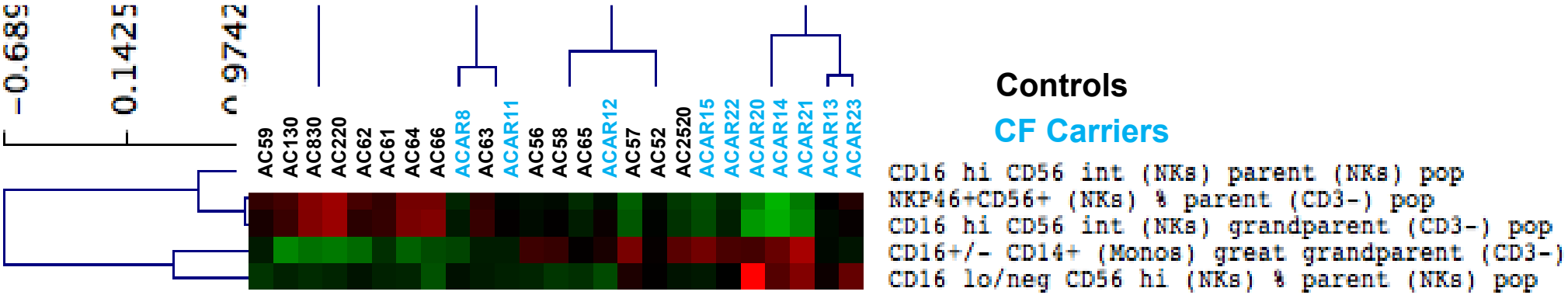

-0.685
0.1425
0.9742
AC59
AC130
AC830
AC220
AC62
AC61
AC64
AC66
ACAR8
AC63
ACAR11
AC56
AC58
AC65
ACAR12
AC57
AC52
AC2520
ACAR15
ACAR22
ACAR20
ACAR14
ACAR21
ACAR13
ACAR23
Controls
CF Carriers
CD16 hi CD56 int (NKs) parent (NKs) pop
NKP46+CD56+ (NKs) % parent (CD3-) pop
CD16 hi CD56 int (NKs) grandparent (CD3-) pop
CD16+/- CD14+ (Monos) great grandparent (CD3-)
CD16 lo/neg CD56 hi (NKs) % parent (NKs) pop

Fig. 24. Heat map of significantly different monocyte, natural killer, dendritic and myeloid derived suppressor cell subset percentages in CF carriers and healthy controls. Raw data were imported in txt. file format to MeV. Data were normalised across rows and a nonparametric Kruskal–Wallis test was performed to determine differences in the proportions of each subset between groups. An unsupervised clustering analysis (hierarchical clustering) was performed using a nonparametric Spearman rank correlation distance metric and average linkage clustering. A heat map expression image was created to visualise results. Rows correspond to the immune cell subsets and columns correspond to individual subjects. Black squares indicate unchanged relative expression, green squares indicate down regulated relative expression and red squares indicate up regulated relative expression of that variable.

Table 23. Relative mRNA expression of immune-related markers in $CD4^+$ cells in CF carriers compared with control and CF groups

	Control	CF	Carrier	Carrier vs Control	Carrier vs CF
				***P* value**	***P* value**
N	16	10			
Age median (range)	28 (23–54)	25.5 (19–55)	32 (24–48)	0.41	0.23
Sex male (%)	6 (37.5%)	5 (50%)	4 (36%)		
Relative mRNA Expression					
TNF median (range)	1.6050 (0.7819–6.3420)	3.0770 (0.9395–11.2000)	3.4343 (1.2968–5.7757)	**0.001**	0.77
PPARγ median (range)	0.0321 (0.0094–0.1012)	0.0611 (0.0030–0.2449)	0.0425 (0.0161–0.1593)	0.47	0.61
PON2 median (range)	0.1024 (0.0439–0.2432)	0.1253 (0.0127–0.5783)	0.0994 (0.0421–0.3855)	0.72	0.43
CCR4 median (range)	0.0369 (0.0077–0.1523)	0.0507 (0.0031–0.1941)	0.0637 (0.0245–0.1975)	0.22	0.68
IL-6 median (range)	0.0130 (0.0069–0.04000)	0.0127 (0.0035–0.0271)	0.0109 (0.0032–0.0145)	**0.019**	0.13
c-REL median (range)	0.0611 (0.0301–0.1107)	0.0412 (0.02936–0.0554)	0.0369 (0.0294–0.0537)	**0.002**	0.23
FOXP3 median (range)	0.0034 (0.0015–0.0082)	0.0027 (0.0008–0.0048)	0.0032 (0.0018–0.0062)	0.73	0.31
T-BET median (range)	0.0064 (0.0016–0.0157)	0.0029 (0.0002–0.0078)	0.0071 (0.0048–0.0125)	0.31	**0.0005**
GATA3 median (range)	0.0057 (0.0039–0.0093)	0.0061 (0.0033–0.0178)	0.0075 (0.0049–0.0187)	0.06	0.15
STAT3 median (range)	0.0299 (0.0131–0.1026)	0.0244 (0.0035–0.0474)	0.0301 (0.0108–0.0524)	0.58	0.92
CXCR3 median (range)	0.0063 (0.0029–0.0137)	0.0044 (0.0019–0.0073)	0.0046 (0.0024–0.0117)	0.39	0.64
c-MAF median (range)	0.0003 (0.0001–0.0009)	0.0002 (0.00002–0.0007)	0.0001 (0.00002–0.0008)	**0.014**	0.73
AHR median (range)	0.0047 (0.0015–0.0167)	0.0060 (0.0033–0.01365)	0.0117 (0.0068–0.0154)	**0.003**	**0.005**
CCR6 median (range)	0.0367 (0.0066–0.1539)	0.0387 (0.0013–0.1044)	0.0387 (0.0092–0.0828)	0.90	0.76

P values indicating significant differences are shown in **bold**

7.4 Discussion

This study is the first to analyse the proportions of peripheral $CD4^+$ effector (Th1, Th2, Th17) and regulatory ($FOXP3^+$ Treg, IL-10^+ Tr1, TGF-β^+ Th3) subsets, innate immune populations such as monocytes, DCs, NKs and MDSCs, and the gene expression of CD4 transcriptional regulators, homing and inflammatory markers in human *CFTR* heterozygotes (CF carriers). It demonstrates for the first time that many peripheral immune cell abnormalities in CF are likely to be related to an inherent defect associated with *CFTR* mutations rather than to pulmonary infection and inflammation, because CF heterozygotes/carriers present with an immune phenotype that appears, in many aspects, similar to that of CF patients or with an intermediate phenotype between that of CF patients and healthy controls, indicating a potential *CFTR* 'dose effect'.

While Th1, Th2 and Th17 proportions did not differ significantly between controls, carriers and CF, the proportions of $FOXP3^+$ Tregs and IL-10^+ Tr1 cells were significantly higher in CF carriers compared with those in healthy controls. $FOXP3^+$ Tregs were also significantly higher in CF carriers compared with CF patients, while IL-10^+ Tr1 cells showed a trend towards an increase. This may suggest that while these regulatory cells are increased as a result of *CFTR* mutations, the presence of chronic infection in CF patients (70% chronic P. aeruginosa) causes a superimposed reduction of these cells although levels in CF patients are still higher than in healthy controls. This is consistent with studies that have shown decreases in Tregs in association with CF-related infections such as *P. aeruginosa* and RSV (217, 365). Increases in regulatory cells in CF carriers may also be a contributing factor to the reduced severity of influenza infection that has been associated with *CFTR* heterozygosity (366), because Tregs are essential for efficient clearance of this infection (367). Interestingly, TGF-β^+ Th3 cells displayed a differing pattern to that of the other regulatory cells, in that this population appeared very similar in CF carriers and controls, but was trending towards being lower in both than in the CF group. This may suggest that the changes seen in this population in CF are indeed related to infection and inflammation rather than specifically to the *CFTR* mutation.

It must be noted that the median age of the CF carrier group for this analysis was significantly higher than that of the CF and control groups. However, studies have shown Tregs decrease rather than increase with age (368, 369), suggesting that the difference in age is unlikely to be responsible for the observed increases in regulatory populations.

With regard to memory $CD4^+$ and Treg cells, CF carriers presented with either a similar phenotype to that seen in CF patients or a phenotype intermediate between CF patients and controls. Although we saw no significant difference in $CXCR3^+$ (Th1-associated) $CD4^+$ or Treg cells in CF carriers compared with either healthy controls or CF patients, the median level in carriers was intermediate between those in the other two groups, indicating a potential role of *CFTR* 'dose' in changes of this population.

Interestingly, we saw a significant decrease in both $CCR6^+$ (Th17-associated) $CD4^+$ and Treg cells in CF carriers compared with healthy controls, a similar trend to that observed in CF patients. This could suggest that either these cells are inherently reduced in the periphery as a result of *CFTR* mutation or potentially that carriers have increased production of Th17-associated chemoattractants, such as the CCL20 that is produced by human airway epithelial cells, as has been shown in CF patients (370), which influences the homing of $CCR6^+$ cells to the lungs. Intriguingly, we saw an increase in naïve $CCR6^+$ Treg cells in the blood of CF carriers compared with that of CF patients. Potentially, the *CFTR* mutation leads to an increase in the proportion of these Th17-associated naive cells but the chronic infection associated with CF may result in their differentiation into effector cells that then home to the lungs, where increased levels have been reported in CF (283).

As seen in CF patients, we see a normal level of $CCR4^+$ $CD4^+$ (Th2-associated) cells in CF carriers but a decrease in $CCR4^+$ Treg cells, indicating the potential for a Th2 bias in CF carriers because there are fewer Th2-associated regulatory cells able to suppress a normal level of Th2-associated effector cells. This may contribute to the increased incidence of asthma that has been reported to be associated with CF heterozygosity (361), because asthma is a known Th2-associated disease.

Innate immune cells, like $CD4^+$ cells, also appear to be affected by defective CFTR to varying degrees. NK cells in CF carriers, both when measured as one heterogeneous population based on NKp46 and CD56 expression and when divided into subpopulations based on CD16 and CD56 expression, displayed a very similar phenotype to that in CF patients. NKp46 NK cells were decreased and there was a shift from $CD56^{int}$ (cytotoxic) to $CD56^{hi}$ (cytokine-producing) NKs compared with healthy controls. NK cells are known to influence adaptive immune responses through interaction with antigen presenting cells such as DCs, activation of T cells through production of cytokines and chemokines, and by killing T cells (371). A reduction in

these cells could therefore have a significant impact on the shape of both the innate and adaptive immune response in CF carriers and could be a contributing factor to the increased frequency of inflammatory illnesses such as asthma and chronic rhinosinusitis that has been associated with *CFTR* heterozygosity (361, 362).

Neither $CD14^{+}$ (monocytic) nor $CD14^{-}$ (granulocytic) MDSCs, highly suppressive innate populations, differed significantly in their proportions in CF carriers compared with those in healthy controls or CF patients, but showed an intermediate phenotype. However, the median percentage for these cell types more closely resembled those of the healthy controls, suggesting that infection and inflammation may also contribute to their altered levels in CF patients, as suggested by Rieber *et al.* (126).

DCs, which are known to express CFTR normally (23) and which are increased in CF, show a trend towards being increased in CF carriers compared with healthy controls. DCs are professional antigen presenting cells and therefore have great influence over the shape of the adaptive immune response. Dysregulation of the adaptive immune response, like the Th2 Th17 biases seen in CF patients, could be a contributing factor toward the increased incidence of illnesses such as bronchiectasis in CF carriers.

Monocytes, which also act as antigen presenting cells and therefore can have considerable influence over the shape of the adaptive immune response, were found to be significantly increased in CF carriers compared with healthy controls, and similar to levels in CF patients. When individual monocyte populations were analysed, 'classical' proinflammatory $CD16^{lo/neg}CD14^{hi}$ monocytes trended towards being higher in CF carriers compared with healthy controls while 'non-classical' (more responsive to viral than bacterial infection) $CD16^{hi}CD14^{lo}$ monocytes did not differ significantly from either the control or CF groups, presenting with an intermediate phenotype. While neither subset was significantly increased, an overall increase in these proinflammatory cells could play an important role in dysregulation of both the innate and adaptive immune responses in CF carriers.

Interestingly, when a heat map of the significantly differing innate immune cell subsets between CF patients, carriers and healthy controls was generated, there was a lack of distinct clustering of the CF carriers. While no clear patterns were present it is clear from the scattered clustering that the featurs of some CF carriers resemble those of CF patients while others are more like healthy controls. It must also be noted that given the 1 in 25 carrier frequency rate, there may be CF carriers within the control population. However, we were unable to identify

any factors to explain this observation. When CF carriers were compared individually to healthy controls and CF patients, using hierarchical clustering and associated heat map, CF carriers' immune phenotype appeared more like that of CF patients than healthy controls. In particular, many of the significantly different subsets identified between CF carriers and healthy controls were NK cells. This may indicate that, of the peripheral immune cells, NK cells are most greatly affected by defective CFTR. The survival of NK cells is promoted by the secretion of IL-15 by bronchial epithelial cells (372) and, given that this cell type is known to normally express CFTR, it may be that dysregulation of the production of this cytokine by dysfunctioning epithelial cells in CF carriers and CF patients is contributing to a reduction of NK cells. Additionally, NK cells can be suppressed by other cells such as alveolar macrophages (136), and given that we have shown dysregulation of monocytes in CF carriers and patients, this may also be a contributing factor to the reduction in NK cells we see in these two groups.

Interestingly, gene expression of the proinflammatory cytokines TNF and IL-6 in CD4 cells of CF carriers was found to be upregulated and downregulated, respectively, compared with controls. Consistent with this, TNF production by T cells has been shown to be increased because of increased nuclear localisation of NFAT as a consequence of *CFTR* mutation (22). Although IL-6 expression is known to be increased in CF, it is possible that this is due to the presence of chronic infection and inflammation and that the effect of *CFTR* mutation is a decreased expression as seen in carriers. IL-6 is also known to downregulate regulatory T cells (373), therefore a decrease in IL-6 and subsequently less suppression of Tregs fits with the flow cytometric data showing increased Tregs in CF carriers.

Expression of *c-REL*, a transcription factor involved in the differentiation of nTregs, was found to be decreased in CF carriers compared with healthy controls, and similar to that seen in CF patients, while expression of *AHR*, a transcription factor involved in the differentiation of iTreg and IL-10^+ Tr1 cells, was upregulated. Therefore, while differentiation of nTregs may be decreased, transcriptional regulation of iTregs and IL-10^+ Tr1 cells is upregulated, as confirmed by the presence of increased iTregs and Tr1 cells analysed by flow cytometry.

Lastly, while *T-BET*, a transcription factor involved in the differentiation of Th1 cells, was significantly decreased in CF patients, it appeared to be expressed at normal levels in CF carriers. This may indicate that the Th2 bias reported in CF is in part related to chronic infection and inflammation.

A major strength of this study is its novelty: it is the first study to analyse immune cell subsets in CF carriers. Another strength is the diverse range of markers investigated in the CF carrier population at both cellular and genetic levels. However, functional testing of these subsets would provide a more complete picture of the CF carrier immune response. While we have shown alterations in the proportions of several immune cell populations in CF carriers, we have not investigated the level or function of CFTR in these cells. However, a study by Sorio *et al.* (311) investigating primary monocytes from CF carriers has shown that they have decreased CFTR function compared with those of healthy controls; it is likely that similar effects would be seen in other immune cells, particularly those also known to normally express CFTR such as DCs and lymphocytes. Lastly, we did not genotype our healthy control population and therefore the possibility exists that some CF carriers may be present within the control population, although given the carrier rate of 1 in 25, this would likely be limited to a maximum of one or two individuals.

In summary, these results suggest that CF carriers experience adaptive and innate immune dysfunction that in many cases is similar to that of CF patients or intermediate between CF patients and healthy controls. This may suggest that *CFTR* heterozygosity may cause subtle changes that are detectable in individual immune subsets, potentially influencing the susceptibility of carriers to development of a number of disorders that research is only just beginning to identify. Further research involving CF carriers is required to determine the full extent of the clinical consequences of *CFTR* heterozygosity. Given that 1 in 25 people in Australia are CF carriers, this could have widespread impact.

This study also demonstrates that defective CFTR may play a much larger role in CF immune pathogenesis than research has previously indicated. In particular, NK cells appear the most greatly affected by defective CFTR. Further investigation into the functional capacity of these immune cells may also lead to the identification of potential therapeutic targets for CF.

CHAPTER 8

General Discussion

Lung infection is the primary cause of morbidity and mortality in CF. There is an ongoing debate as to whether infection or inflammation occurs first in the lungs of people with CF as opposed to inflammation being strictly a consequence of infection. The conventional theory (Fig. 25) proposes that defective CFTR results in an accumulation of mucus that promotes infection and then consequently inflammation that is damaging to the lungs. However studies are emerging that have shown increased levels of neutrophils in BALF from young CF patients without detectable infection (30, 31) as well as increased macrophages in CF foetal lungs, although there is also evidence to suggest that neutrophil levels are normal in BALF from 'infection-naïve' CF children (41). However, the evidence (256, 257, 356, 374-376) to support an inherently proinflammatory immune state in CF is strong, suggesting that defective CFTR results directly in both an increase of lung-damaging proinflammatory immune cells and in an accumulation of mucus, and that infection simply exacerbates these (Fig. 25). Our research also supports this concept as we have shown alterations in levels of both innate and adaptive immune cells in CF carriers who do not experience any serious infection such as those seen in people with CF disease. This demonstrates the important role CFTR plays in maintaining appropriate immune cell levels, but leaves open the question of how their function may also be altered. Current knowledge about innate and adaptive immune cells in CF patients and carriers is summarised in Table 24.

Table 24. A summary of the known alterations in innate and adaptive immune cells in CF carriers and patients.

Cell Type	Alteration in CF Carriers	Reference	Alteration in CF	Reference
Neutrophils	Reduced apoptosis	(377)	Increased pulmonary levels Decreased microbicidal activity Reduced phagocytic capacity Increased elastase secretion Enhanced chemotaxis Reduced apoptosis Altered cytokine production	(100) (96-98) (99) (251) (252) (253, 254) (255)
Macrophages	Increased pulmonary levels	(366)	Increased pulmonary levels Altered cytokine production Reduced antigen presentation capacity	(102) (102, 257) (258)
Monocytes	Altered cytokine secretion Increased peripheral blood levels of monocytes (measured as one heterogeneous population) Trend towards increased 'classical' $CD14^{hi}CD16^{lo/neg}$ monocytes Peripheral blood 'non-classical' $CD14^{lo}CD16^{hi}$ level intermediary to CF and healthy controls	(88) This study This study This study	Increased 'classical' $CD14^{hi}CD16^{lo/neg}$ and 'non-classical' $CD14^{lo}CD16^{hi}$ monocytes Reduced phagocytosis Increased elastase secretion Decreased adhesion Altered cytokine production Reduced antigen presentation capacity	This study (259) (90) (85) (88) (260)
Dendritic cells	Trend toward increased levels Peripheral blood level intermediary to CF and healthy controls	This study This study	Increased peripheral blood levels Altered membrane structure Delayed differentiation Decreased antigen presentation	This study (23) (23, 261) (114)
Natural killer cells	Increased peripheral blood levels of NKs (measured as one heterogeneous population) Switch from $CD56^{int}$ to $CD56^{hi}$ NK peripheral blood phenotype	This study This study	Decreased peripheral blood levels Switch from $CD56^{int}$ to $CD56^{hi}$ NK peripheral blood phenotype	(138-140), This study This study
Myeloid derived suppressor cells	Peripheral blood levels of $CD14^{-}$ and $CD14^{+}$ MDSCs intermediary to CF and healthy controls	This study	Switch from $CD14^{-}$ to $CD14^{+}$ peripheral blood phenotype	This study
T Lymphocytes	Increased regulatory (FOXP3+ Treg & IL-10^{+}Tr1) cells Decreased peripheral blood levels of memory Tregs Decreased peripheral blood levels of memory Th17 (CCR6) associated $CD4^{+}$ cells Peripheral blood levels of memory Th1 (CXCR3) associated $CD4^{+}$ & Tregs intermediary to CF and healthy controls Decreased memory Th2 (CCR4) associated Tregs	This study This study This study This study	Th2 & Th17 bias Altered cytokine production (CD4 & CD8) Increased peripheral blood levels of regulatory (FOXP3+ Treg, IL-10^{+} Tr1, TGFβ^{+} Th3) cells Decreased peripheral blood levels of memory Th1 (CXCR3) and Th17 (CCR6) associated $CD4^{+}$ cells Decreased peripheral blood levels of memory Th1 (CXCR3), Th2 (CCR4), Th17 (CCR6) associated Treg cells	(53, 262), This study (22, 115, 263) This study This study This study
B Lymphocytes			Altered Immunoglobulin production Altered differentiation	(145, 146, 149, 150) (144)

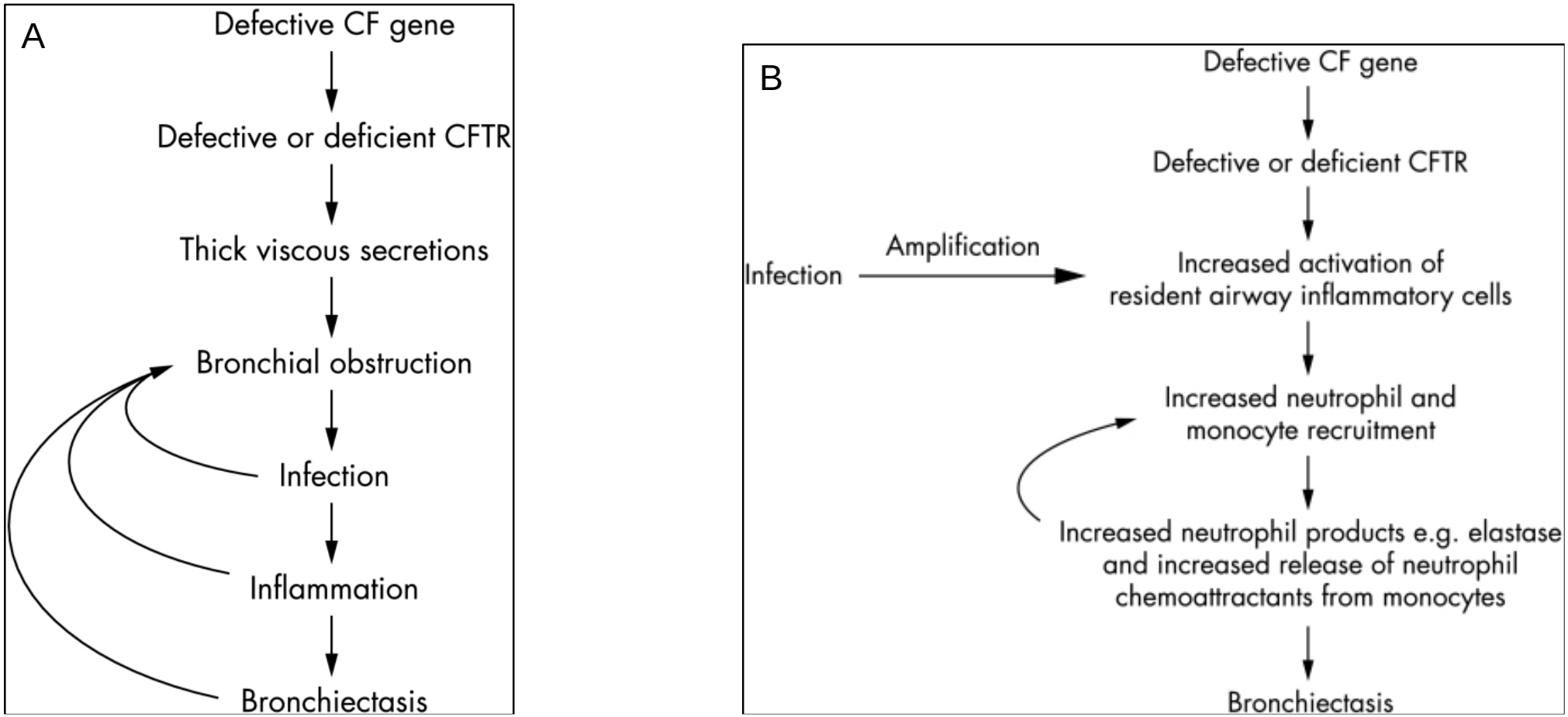


Fig. 25. Traditional versus modern view of CF lung lung disease pathology. 'A' represents the traditional view of mucus-mediated lung pathology while 'B' represents the modern inflammatory cell-mediated view. Figure taken from Rao and Grigg.

CFTR has been shown to be expressed by innate immune cell populations such as neutrophils (96), monocytes, macrophages (311) and DCs (23) and adaptive immune cells such as lymphocytes (22). We investigated a diverse range of innate and adaptive immune cell populations in the peripheral blood of people with CF, away from the main site of infection in the lungs, to reduce the bias in populations resulting from chronic and acute infections. Our analyses have demonstrated that immune dysregulation in CF is widespread, affecting both the innate and adaptive immune responses. By including in the analyses CF carriers and children with CF, we have also provided evidence to suggest that many of the immune abnormalities observed in CF are a result of defective CFTR rather than simply the presence of infection. This therefore supports the emerging theory that lung damage in CF is a consequence of both CFTR immune dysfunction and of infection rather than being purely infection-related. In support of this, we have shown a negative correlation between Th17% in peripheral blood and FEV1 % predicted, not only in CF adults but also in paediatric CF patients who presented with no detectable infection. This therefore indicates that even prior to any infection these cells are inherently damaging to the lungs. In support of this a study by Bratcher *et al.* (378) has also shown that treatment of CFTR monocytes with a CFTR corrector results in improved functioning. In addition, Virella-Lowe *et al.* (379) have shown, using CF bronchial epithelial cell lines, that defective CFTR results in altered expression of up to 843 genes, indicating an extensive role for CFTR in cellular processes additional to its activity as a chloride ion channel. We have also shown that the global immune phenotype of CF carriers is closer to that of CF patients than to that of healthy controls, with some features indicating an intermediate phenotype possibly related to gene dosage. This intermediate phenotype was identified when the median level of an immune cell subset in CF carriers did not differ significantly from that of CF patients or healthy controls while controls and CF patients differed significantly from each other. This may indicate that, in these intermediate cases, possessing just one functional *CFTR* allele is adequate to result in sufficient immune cell levels/function and may suggest why CF carriers do not display the severe clinical consequences seen in those with two dysfunctional copies of *CFTR*. The immune alterations we see in the CF carrier population likely provide the basis for the reported increased incidences of immune related illnesses such as asthma in this group. However, further studies are required to determine the full extent of the effects of immune-related changes on disease development in CF carriers and if intervention is required or would be beneficial.

We have shown that while there are subtle changes in the adaptive immune responses of people with CF, including increased proportions of $FOXP3^+$ Tregs, IL-10^+ Tr1 and TGF-β^+ Th3 regulatory cells and decreased proportions of $CXCR3^+$ (Th1-associated), $CCR4^+$ (Th2-associated) and $CCR6^+$ (Th17-associated) $CD4^+$ and Treg cells, the changes in innate immune cells are much more striking. While MeV analyses were performed on adaptive T cell data and heat maps generated (data not shown), clear differences like those seen in the innate immune cell MeV analyses were not identified, supporting the subtler nature of these changes in the adaptive immune cells. Aberrations were seen in all innate immune populations measured, including monocytes, DCs, NKs and MDSCs, with the greatest effect being seen in NK cells. These innate immune cell types are the first line of defence against pathogens and can influence the adaptive response primarily through antigen presentation and secretion of cytokines and chemokines. DCs and monocytes/macrophages in particular can have great influence over the shape of the adaptive response though antigen presentation. Therefore, the changes seen in adaptive $CD4^+$ cell populations may be the result of innate immune cell dysfunction, possibly as a direct consequence of CFTR dysfunction, and not only the result of mutated *CFTR* in the T cells themselves. While there are no studies in CF specifically investigating the effect of innate immune cell function on adaptive immune responses, there is evidence demonstrating inherent CFTR-associated defects in cell types such as macrophages (256, 257, 259), monocytes (92) and DCs (23), all of which have considerable influence over the shape of the adaptive immune response. In particular, Del Fresno *et al.* (260) have demonstrated impaired antigen presentation capacity in CF monocytes, which could be a contributing factor behind the altered levels of adaptive immune cells we recorded.

However, ours is the first study to indicate such global alterations in CF immune cells. We have investigated many of the key cell types in CF, with the exception of neutrophils, macrophages, B cells and $CD8^+$ T cells, although evidence suggests that these populations are also inherently affected by dysfunctional CTFR. This suggests that, because of the highly interrelated nature of the immune system, immune responses in CF are inherently altered all the way from the initial innate immune response through to the complex adaptive responses. Given the nature of this proposed CFTR-dependent immune dysfunction in CF, even a partial correction of CFTR function may decrease inflammation and therefore lung damage in patients, irrespective of any reduction in infection. Neutrophils and macrophages were not included in our analyses as many studies have previously characterised their role in CF. We also did not investigate B cells and $CD8^+$ T cells as the primary focus of our study was the

$CD4^+$ T cells and innate immune cells which subsequently influence the $CD8^+$ T cell and B cell responses through antigen presentation and T cell-dependent activation (142). However, given the minimal research involving B cell responses as well as the dysregulation of T cells and innate immune cells that we have shown, this could prove to be an interesting population to investigate in the future. In addition, while we have investigated many of the known T cell subsets, other minor and more recently described T cell populations such as T follicular helper/regulatory cells, NKT cells, γδ T cells and Th9 cells could be additionally contributing to the pathogenesis of CF, as new research has begun to suggest (380-382). For instance, while NKT cells have been shown to reduce autoimmune responses in CF they have also been shown to contribute to the inflammatory response via production of IL-17, which attracts neutrophils and macrophages whose products are damaging to the lungs (382). Th9 cells have also been implicated in lung inflammation through exacerbation of allergic inflammation via production of IL-9 that promotes mast cell cytokine secretion (381).

When considering adaptive immune responses in people with CF, a Th2 bias has been reported, particularly in response to pathogens such as *P. aeruginosa* and *A. fumigatus* (52, 262). We have identified many immune abnormalities that could be contributing to this phenomenon, regardless of infection status. Decreases in $CD4^+IFN\gamma^+$ (Th1 cells), memory $CXCR3^+$ $CD4^+$ (Th1-associated) and Treg cells together with decreases in gene expression of *T-BET* (required for Th1 differentiation) and *c-REL* (involved in IFNγ production by T cells), all of which are associated with Th1 cells, were recorded. In addition, $CCR4^+$ memory Tregs (Th2-associated) were decreased but $CCR4^+$ memory $CD4^+$ cells were not. A previous study has also shown that in the presence of high levels of IL-10 (which we found to be increased in CF $CD4^+$ cells), DCs tend to prime naïve $CD4^+$ T cells to secrete IL-4, a Th2-associated cytokine (383). This Th2 bias is significant because research has shown that Th1-mediated immune responses are better for clearance of many pathogens, particularly *P. aeruginosa*, and improved lung function (262). This is also supported by our data that revealed a negative correlation between $CCR4^+$ (Th2-associated) memory $CD4^+$ and Treg cells and lung function as well as a positive correlation between the $CD4^+$ CXCR3(Th1)/CCR4(Th2) ratio and lung function. Therefore, enhancement of Th1-mediated immune responses, which has yet to be attempted in CF, could be very advantageous for reducing morbidity and maintaining lung function.

We have also shown that the proportions of both adaptive and innate immune cells including Th17, NK cells and MDSCs are correlated with FEV1 % predicted, indicating that both innate

and adaptive immune responses play a significant role in CF pathogenesis. This is also the first demonstration that peripheral immune cell subsets can reflect lung pathology in CF and therefore could be useful surrogate markers of lung function decline. They could also be used as a marker of efficacy during therapeutic intervention trials, especially with the emerging trend towards the development of targeted CFTR correctors, such as Ivacaftor and Orkambi, although these have yet to be validated in relation to correcting, or at least not exacerbating, immune function deficits. Our data have demonstrated that NK cells appear most affected by CFTR dysfunction, being reduced in both CF patients and carriers, which may indicate that therapies focussed on increasing NK cells may be beneficial. While there are studies that have aimed to therapeutically increase NK cells for treatment of diseases such as cancer, this is logistically difficult because of the low abundance of NK cells. However, researchers have been able to expand NK cells *ex vivo* by stimulation with cytokines and stimulatory cells, and then transfuse these back into the body where they can further proliferate *in vivo* (384). Emerging therapies such as this, although designed for other conditions, may be beneficial for those with CF.

It must be noted that nearly all of our adult CF patients are administered antibiotics, mostly including the potent anti-inflammatory azithromycin. Corticosteroids are also potent anti-inflammatories, although very few of our patients are treated with these. However, although no correlation was found between any antibiotic/steroid treatment and changes in any immune population, it must be considered that the immune phenotype identified in CF patients may be affected to some degree by administration of these drugs. In particular, azithromycin has previously been shown to affect the expression of NFκB (385), which is involved in the regulation of many immune processes, particularly in T cells (386). It was therefore important to explore all immune markers in CF children and carriers who are rarely if ever treated with such drugs. In addition to this, given the extent of immune dysfunction apparent in CF, the nature of treatment with immunosuppressants following lung transplantation in CF must be reflected upon. It has been shown that CF patients receiving lung transplantation, especially those with reduced lung function, have only a 50% survival rate two years post-transplant (387) compared with non-CF individuals who have an 87% and 68% survival rate one and three years, respectively, post-transplant (388). It is possible that greater understanding of the underlying CF immune dysfunction could result in improved treatment regimens following lung transplantation being tailored to CF patients.

Additionally, it must also be considered that immune dysfunction may be playing a role in many common manifestations of CF that have not traditionally been associated with the immune response, for instance pancreatitis and diabetes. Pancreatitis has recently been shown to be associated with increases in NFAT (389), which is a transcription factor revealed to be increased in CF (22). Diabetes is also known to be associated with alterations in immune cell chemotaxis, phagocytosis and cytokine production (390) which have all been recognized to be affected in CF. However, further investigation would be required to determine the involvement, if any, of the immune system dysfunction in these CF-associated manifestations.

Interestingly, we saw increases in peripheral blood Tregs defined by $CD4^+FOXP3^+$ staining but no change in $CD4^+CD25^+CD127^+$ Tregs in CF adults compared with healthy controls. This highlights the possibility that, because we did not include the additional CD25 marker in the first analysis, other $CD4^+$ populations such as T follicular regulatory and γδ T cells that can express FOXP3 (296, 297) may have been included in our initial analysis. This indicates that there are likely to be changes in other minor regulatory populations in CF, which should be further investigated. It also indicates that, as suggested by Fazekas de st Groth *et al.* (199), the more detailed subset analysis described in Chapter 4 may be superior to that described in Chapter 3 for identifying a homogeneous population of Tregs, especially as it does not require permeabilisation of the cells.

While we have shown abnormalities in the proportions of many immune cell populations we have not assessed their function, other than the ability of $CD4^+$ cells to produce their signature cytokines after nonspecific stimulation. While initial studies examining the function of some cell types such as Tregs (217), monocytes (92), macrophages (259), DCs (114) and neutrophils (96-98) have been undertaken in CF patients, there has been no investigation into the functionality of more specific subsets within these populations. Given the small numbers of some of these subsets and therefore the difficulty of isolating sufficient numbers for detailed analysis, assessment of the functionality of these subsets would most likely need to be undertaken by identifying functionality-related markers using flow cytometry or through high-sensitivity techniques such as RNAseq. However, a disadvantage of this latter technique is that gene expression does not always correlate with protein expression (391), as we have shown with our data. For example, while the proportion of cells expressing FOXP3 protein was increased in $CD4^+$ cells, *FOXP3* gene expression was unchanged.

While the intention of this study was to identify inherent immune defects in people with CF and therefore was focused on cells in peripheral blood away from the main site of infection,

investigation of the same cells in the lungs at the site of infection and in the lymph nodes at the site of antigen presentation may, when combined with functional studies, provide a greater overall understanding of immune dysfunction in CF. However, procurement of these types of samples is quite difficult and they usually only become available in end-stage disease, which would not necessarily be reflective of the whole CF population. Investigation of plasma cytokine levels may also be informative, because we have only investigated the specific cell types that produce particular cytokines. Measurement of plasma cytokine levels would allow us to detect signature cytokines produced by all cell types, including ones we did not investigate.

In conclusion, we have defined, in the greatest detail to date, the immune phenotype of people with CF and that of CF carriers. While the dysfunctional immune response is rarely acknowledged when the primary manifestations of CF are discussed, this study provides evidence that greater importance should be placed on this aspect of CF, particularly as the immune response is systemic. Fig. 26 provides an overview of the primary manifestations of CF updated to include the findings of this study. The findings of our research suggest that many immune abnormalities in CF are indeed associated with *CFTR* mutation rather than being simply the result of the presence of infection and inflammation. The implication of this finding could be a move towards a greater focus on treating immune defects in CF in addition to targeting infection. It is possible that the wave of therapeutic CFTR correctors currently entering the market for treatment of CF could also correct the CF immune response, ultimately preserving lung function irrespective of infection status and leading to greater quality of life and life expectancy. These findings could therefore be useful as a means of long-term monitoring and management of patients that are currently on correctors. Furthermore, with the estimated 1 in 25 carrier rate of CF (392), up to one million of the 23+ million people in Australia could potentially be affected by a subtle as yet unrecognised immune dysfunction, the consequences of which are only just beginning to be uncovered.

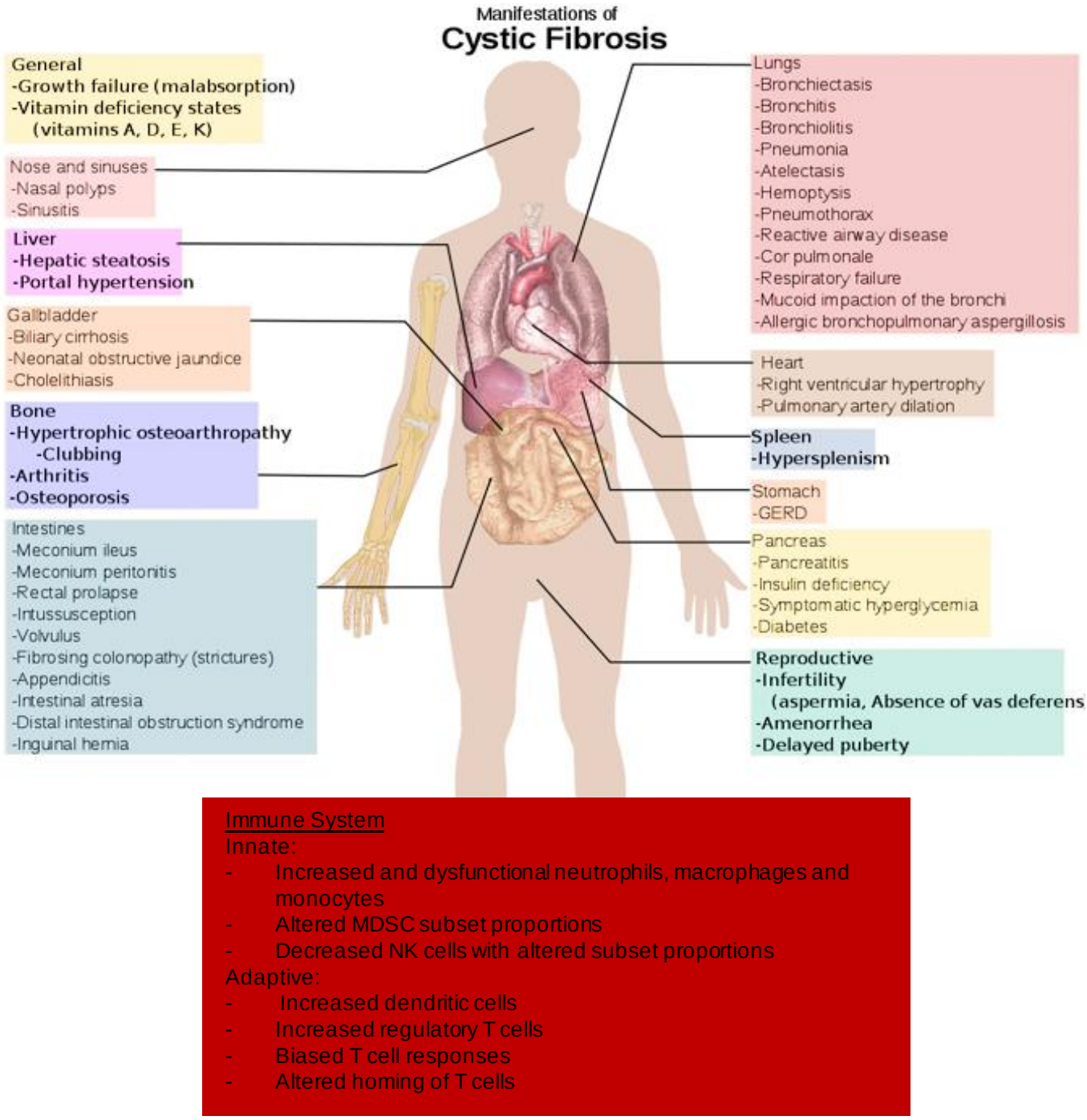


Fig. 26. The primary manifestations associated with CF. Figure modified from Willacy *et al.* (393).

Future Prospects

While we have now demonstrated extensive alterations in the immune system of CF carriers, it would be valuable to investigate the level and function of CFTR in CF carriers. While a small study involving chloride-sweat testing of four CF carriers has shown that they on average have 50% functionality of CFTR compared with that of healthy controls (394), it would be prudent to confirm this with a greater participant pool involving all *CFTR* mutation classes and using more specific tests for protein channel functionality such as that described by Sorio *et al.* (311) in which immune cells were treated with CFTR stimulants and inhibitors whilst membrane potential was measured. In addition, it would be important to determine the effect of CFTR correction on the immune system. Normalisation of the immune system would confirm a direct role of CFTR dysfunction in dysregulation of the CF immune response. This could be undertaken through studying the effects of CFTR correctors, such as Ivacaftor and Orkambi, on CF and CF carrier immune cells (*in vitro* and *in vivo*) or in *cftr* knockout mice to determine any modulations that occur in the level and function of immune cells. It would also be particularly interesting to undertake longitudinal studies not only of CF patients to see how these immune populations change over time with progression of the disease, but also of CF carriers to see how the levels/function of these cells change not only over time but also in relation to the development of any clinical conditions that may not necessarily been associated previously with reduced function of CFTR.

APPENDICES

1. Buffers and Media

1.1 Complete Culture Media

RPMI media
5% Foetal Calf Serum
1% Penicillin–Streptomycin
1% L-glutamine

1.2 FACS Buffer

1.5 L 1× Phosphate Buffered Saline
1.5 g Bovine Serum Albumin
3 ml 10% Sodium Azide

1.3 Cell Sorting Media

1× Phosphate Buffered Saline
2% Foetal Calf Serum
1mM EDTA

1.4 BL + TG buffer

Per sample:
500 μL BL buffer
5μL 1 thioglycerol

2.1 Patient Characteristics:

2.1.1 CF Adults

Table 25. Clinical characteristics of adult CF patients

	Age	Sex	Genotype (no. of DF508 alleles)	Infection	Chronic *P. aeruginosa* infection	*S. aureus* infection	*A. fumigatus* infection	Other infection	Past *A. fumigatus* culture	ABPA
AP 3(3)	25	N	2	N	N	N	N	N	N	N
AP 7(2)	23	N	1	Y	N	Y	N	Y	Y	N
AP 14(2)	21	N	0	Y	Y	Y	N	N	Y	N
AP 16	31	N	2	Y	Y	N	N	N	N	N
AP 16(2)	31	N	2	Y	Y	N	N	N	N	N
AP 17(2)	19	Y	1	Y	Y	Y	N	N	Y	N
AP 18	38	N	2	Y	Y	N	N	N	N	N
AP 22	30	N	2	Y	N	Y	N	Y	N	N
AP 23	24	Y	1	Y	N	Y	Y	Y	Y	N
AP 9(2)	23	Y	1	Y	Y	Y	N	N	Y	N
AP 11(3)	54	Y	2	Y	Y	N	N	N	Y	N
AP 11(4)	55	Y	2	Y	Y	N	Y	Y	Y	N
AP 18(2)	39	N	2	Y	Y	Y	N	Y	N	N
AP 20	38	N	2	Y	N	Y	N	Y	N	N
AP 21(2)	27	Y	1	Y	N	Y	N	Y	N	N
AP 24	22	Y	0	Y	Y	N	N	N	N	N
AP 25	27	Y	2	Y	Y	unknown	Y	unknown	Y	Y
AP 26	52	Y	1	Y	N	Y	N	N	Y	Y
AP 27	24	N	0	Y	Y	N	N	N	N	N
AP 28	24	N	2	Y	Y	Y	N	unknown	N	N
AP 28 (2)	24	N	2	Y	Y	Y	N	unknown	N	N

ABPA: allergic bronchopulmonary aspergillosis; (x): indicates the number of times a patient has provided a sample (i.e AP7(2) indicates that this is the second sample provided by AP7)

Table 26. Clinical characteristics of adult CF patients

	Antibiotics	Azithromycin	Tobramycin	Other antibiotics	Steroids	Exacerbating/ Stable	Total serum IgE (kU/L)	WCC (/nL)	CRP (mg/L)	ESR (mm)	FEV1 % predicted
AP 3(3)	N	N	N	N	Y	Stable	–	5.0	0.05	10.0	87.00
AP 7(2)	Y	N	N	Y	N	Stable	25	15.7	2.50	–	81.98
AP 14(2)	Y	Y	N	Y	N	Stable	–	–	–	–	57.71
AP 16	Y	Y	N	Y	N	Exacerbating	74	12.7	–	–	54.92
AP 16(2)	Y	N	N	Y	N	Stable	–	7.7	16.60	37.0	–
AP 17(2)	Y	Y	Y	N	N	Stable	–	5.4	2.20	11.0	–
AP 18	Y	N	Y	Y	N	Exacerbating	22	6.4	27.10	–	28.15
AP 22	Y	N	Y	Y	N	Exacerbating	–	7.6	6.20	37.0	42.43
AP 23	Y	Y	N	Y	N	Stable	237	3.7	7.30	15.0	63.60
AP 9(2)	Y	Y	N	Y	N	Stable	–	6.1	1.40	5.0	–
AP 11(3)	Y	Y	Y	Y	N	Stable	–	9.0	35.20	59.0	22.99
AP 11(4)	Y	Y	Y	Y	N	Exacerbating	–	5.0	–	–	–
AP 18(2)	Y	Y	N	N	N	Stable	30	8.0	19.90	–	57.19
AP 20	Y	N	N	Y	Y	Exacerbating	–	6.0	23.40	–	–
AP 21(2)	Y	N	N	Y	N	Exacerbating	–	8.4	3.40	–	78.43
AP 24	Y	Y	Y	N	N	Stable	–	5.4	7.00	3.9	–
AP 25	Y	Y	N	N	N	Stable	1535	5.3	3.70	6.0	51.92
AP 26	Y	N	N	Y	N	Stable	–	8.8	1.20	6.0	31.58
AP 27	Y	N	N	Y	N	Stable	–	5.8	15.90	34.0	38.95
AP 28	Y	Y	Y	N	N	Exacerbating	–	13.2	8.30	–	54.94
AP 28 (2)	Y	Y	Y	N	N	Stable	–	5.1	1.20	–	57.44

WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; FEV1: forced expiratory volume in 1 second; (x): indicates the number of times a patient has provided a sample (i.e AP7(2) indicates that this is the second sample provided by AP7)

2.1.2 CF Children

Table 27. Clinical characteristics of paediatric CF patients

	Age	Sex	Genotype (no. of DF508 alleles)	Infection	Chronic *P. aeruginosa* infection	*S. aureus* infection	*A. fumigatus* infection	Other infection	ABPA
Ch.P 1	11.0	M	1	Y	N	N	N	Y	N
Ch.P 2	5.0	F	0	Y	N	Y	N	N	N
Ch.P 3	15.0	F	1	Y	N	N	Y	Y	N
Ch.P 4	11.0	F	1	N	Y	N	N	N	N
Ch.P 5	12.0	M	2	Y	N	Y	Y	N	N
Ch.P 6	14.0	F	1	Y	Y	N	N	N	N
Ch.P 7	1.0	F	2	Y	N	N	N	Y	N
Ch.P 8	4.0	F	0	N	N	N	N	N	N
Ch.P 9	15.0	M	2	Y	N	Y	N	Y	N
Ch.P 10	10.0	M	1	Y	N	N	Y	Y	Y
Ch.P 11	3.0	F	2	Y	N	Y	N	Y	N
Ch.P 12	4.0	F	1	Y	N	N	N	Y	N
Ch.P 13	17.0	F	1	Y	Y	N	N	Y	N
Ch.P 14	15.0	M	1	N	N	N	N	N	N
Ch.P 15	6.0	F	2	Y	N	Y	N	N	N
Ch.P 16	11.0	M	2	N	N	N	N	N	Y
Ch.P 17	6.0	F	0	Y	N	Y	N	Y	N
Ch.P 18	13.0	F	1	Y	N	N	Y	Y	Y
Ch.P 19	11.0	F	2	Y	N	Y	N	N	N
Ch.P 20	15.0	M	2	N	N	N	N	N	N
Ch.P 21	14.0	M	2	Y	N	N	Y	Y	Y
Ch.P 22	0.5	F	2	N	N	N	N	N	N
Ch.P 23	3.0	F	1	N	N	N	N	N	N

ABPA: allergic bronchopulmonary aspergillosis

Table 28. Clinical characteristics of paediatric CF patients

	Antibiotics	Azithromycin	Tobramycin	Other antibiotics	Steroids	Exacerbating/ Stable	Total serum IgE (kU/L)	WCC (/nL)	CRP (mg/L)	FEV1 % predicted
Ch.P 1	N	N	N	N	N	Stable	–	5.0	0.0	–
Ch.P 2	N	N	N	N	N	Stable	–	10.3	0.3	–
Ch.P 3	N	N	N	N	N	Stable	–	–	2.4	–
Ch.P 4	N	N	N	N	N	Stable	–	5.1	–	–
Ch.P 5	Y	N	N	Y	N	Stable	–	7.0	1.5	97.68
Ch.P 6	Y	Y	Y	Y	N	Stable	–	15.2	1.6	51.07
Ch.P 7	N	N	N	N	N	Stable	–	8.0	–	–
Ch.P 8	N	N	N	N	N	Stable	–	8.9	–	–
Ch.P 9	N	N	N	N	N	Stable	–	11.4	–	–
Ch.P 10	Y	N	N	Y	Y	Stable	–	8.4	0.2	65.74
Ch.P 11	N	N	N	N	N	Stable	–	13.5	2.3	–
Ch.P 12	N	N	N	N	N	Stable	–	8.6	0.4	–
Ch.P 13	Y	Y	Y	N	N	Stable	–	9.3	10.6	36.35
Ch.P 14	N	N	N	N	N	Stable	–	6.3	0.2	103.64
Ch.P 15	N	N	N	N	N	Stable	–	12.5	0.2	–
Ch.P 16	N	N	N	N	N	Stable	2.86	7.1	0.2	–
Ch.P 17	N	N	N	N	N	Stable	–	8.7	–	–
Ch.P 18	N	N	N	N	N	Stable	13	8.7	0.2	106.21
Ch.P 19	N	N	N	N	N	Stable	–	–	–	–
Ch.P 20	N	N	N	N	N	Stable	–	–	–	117.56
Ch.P 21	N	N	N	N	Y	Stable	17.6	–	–	77.69
Ch.P 22	N	N	N	N	N	Stable	–	–	–	–
Ch.P 23	N	N	N	N	N	Stable	–	–	–	–

WCC: white cell count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; FEV1: forced expiratory volume in 1 second

2.2 Adult CF Carrier Characteristics:

Table 29. Clinical characteristics of adult CF carriers

	Age	Sex	Genotype
A.Car 1	37	F	unknown
A.Car 2	28	F	deltaF508
A.Car 3	43	M	deltaF508
A.Car 4	41	F	unknown
A.Car 5	44	M	deltaF508
A.Car 6	45	F	unknown
A.Car 7	43	F	unknown
A.Car 8	38	F	W1282
A.Car 9	25	M	unknown
A.Car 10	24	F	unknown
A.Car 11	39	F	deltaF508
A.Car 12	45	F	unknown
A.Car 13	32	M	deltaF508
A.Car 14	31	F	deltaF508
A.Car 15	46	M	unknown
A.Car 16	40	F	deltaF508
A.Car 17	29	F	unknown
A.Car 18	46	F	deltaF508
A.Car 19	48	M	deltaF508

3. All flow cytometric data comparing healthy controls, CF carriers and CF patients:

3.1 $CD4^+$ effector (Th1, Th2, Th17) and regulatory ($FOXP3^+$ Treg, IL-10^+ Tr1, TGF-β^+ Th3) T cell subsets

Table 30. CD4+ T cell subset percentages in CF carriers compared with control and CF groups

	Control	Cystic Fibrosis	Carrier	Carrier vs Control *P* Value	Carrier vs CF *P* Value
N	51	20	13		
Age median (range)	22 (18–61)	24 (18–53)	39 (24–45)	**0.002**	**0.018**
Sex male (%)	24 (47%)	12 (60%)	3 (23%)		
$CD4^+$% median (range)	50.40 (29.7–66.0)	48.15 (28.3–66.1)	55.2 (37.6–75.7)	0.051	**0.04**
Th1% median (range)	9.6 (3.0–22.4)	10.45 (3.5–24.6)	11.1 (5.25–29.2)	0.45	0.34
Th2% median (range)	2.6 (0.7–9.8)	2.3 (0.4–8.5)	1.9 (1.19–4.61)	0.60	0.91
Th1/Th2 ratio median (range)	4.6 (1.3–11.3)	5.15.1 5.1 (1.0–10.4)	4.66 (1.89–20.56)	0.50	0.41
Th17% median (range)	0.49 (0.15–2.26)	0.52 (0.04–1.98)	0.73 (0.13–1.19)	0.76	0.88
Treg% median (range)	5.5 (1.8–10.5)	6.46 (4.2–11.4)	8.8 (4.67–14.3)	**0.0004**	**0.03**
Treg/Th17 ratio median (range)	10.6 (2–37)	14.8 (4–441)	12.14 (5.46–102.3)	0.89	0.71
IL-10^+ Tr1% median (range)	1.15 (0.35–2.63)	1.77 (0.41–4.16)	2.74 (0.28–7.97)	**0.001**	0.08
TGF-β Th3% median (range)	0.022 (0.008–0.19)	0.093 (0.018–0.417)	0.03 (0.005–0.40)	0.3	0.08

P values indicating significant differences are shown in **bold**

3.2 CCR4-, CCR6- and CXCR3-expressing CD4+ T cells and Treg cells

Table 31. Percentages of CCR4-, CCR6- and CXCR3-expressing $CD4^+$ T cells and Treg cells in CF carriers compared with control and CF

	Control	CF	Carrier	Carrier vs Control *P* value	Carrier vs CF *P* value
N	15	17	10		
Age median (range)	28 (22–50)	25 (19–54)	34 (22–47)	0.27	0.14
Sex male (%)	5 (33%)	8 (47%)	3 (30%)		
$CD4^+$ %* median (range)	34.00 (20.40–54.10)	34.60 (21.30–59.30)	40.85 (26.10–56.10)	0.23	0.23
$CD4^+RO^+$ %* median (range)	40.50 (19.90–58.40)	30.80 (15.00–61.00)	32.70 (17.00–55.10)	0.49	0.93
$CD4^+RO^-$ %* median (range)	59.50 (41.60–80.10)	69.20 (39.00–85.00)	67.30 (44.90–83.00)	0.49	0.93
$CD4^+CXCR3^+RO^+$ %* median (range)	18.40 (4.13–36.40)	13.20 (6.40–24.50)	14.85 (6.25–23.80)	0.10	0.85
$CD4^+CXCR3^+RO^-$ %* median (range)	2.54 (1.20–12.20)	2.53 (1.25–7.12)	3.14 (0.70–7.06)	0.29	0.44
$CD4^+CCR4^+RO^+$ %* median (range)	23.20 (7.90–38.60)	17.90 (9.69–38.30)	18.75 (11.20–39.30)	0.46	0.53
$CD4^+CCR4^+RO^-$ %* median (range)	1.53 (0.21–5.90)	1.22 (0.19–4.32)	2.34 (0.17–7.77)	0.50	0.29
$CD4^+CCR6^+RO^+$ %* median (range)	17.40 (7.43–34.70)	13.40 (2.94–19.10)	10.25 (4.28–26.90)	**0.026**	0.48
$CD4^+CCR6^+RO^-$ %* median (range)	0.33 (0.15–1.01)	0.30 (0.07–0.72)	0.44 (0.09–1.33)	0.61	0.13
Treg %* median (range)	7.33 (4.00–8.91)	7.85 (5.29–16.30)	7.43 (4.83–9.71)	0.95	0.30
Treg RO^+ %* median (range)	57.10 (40.30–77.60)	51.10 (30.40–91.30)	42.80 (35.80–80.00)	**0.026**	0.54
Treg RO^- %* median (range)	42.90 (22.40–59.70)	48.90 (8.72–69.60)	57.20 (20.00–64.20)	**0.026**	0.54
Treg $CXCR3^+RO^+$ %* median (range)	17.20 (2.81–30.10)	11.70 (3.30–21.20)	12.25 (4.27–26.40)	0.19	0.90
Treg $CXCR3^+RO^-$ %* median (range)	2.74 (1.00–8.14)	3.96 (0.71–7.22)	3.70 (1.40–7.38)	0.26	0.90
Treg $CCR4^+RO^+$ %* median (range)	50.00 (34.10–72.30)	44.90 (21.20–70.80)	40.70 (33.60–77.30)	**0.039**	0.93
Treg $CCR4^+RO^-$ %* median (range)	1.55 (0.53–7.08)	1.96 (0.46–8.76)	3.63 (0.38–12.20)	0.20	0.36
Treg $CCR6^+RO^+$ %* median (range)	33.70 (14.90–48.50)	21.70 (4.20–35.90)	16.15 (7.71–54.90)	**0.023**	0.64
Treg $CCR6^+RO^-$ %* median (range)	0.87 (0.33–2.67)	0.64 (0.12–3.20)	1.36 (0.29–2.48)	0.37	**0.036**
$CD4^+$ $CD45RO^+$ CXCR3/CCR4 ratio median (range)	0.82 (0.18–1.51)	0.67 (0.15–1.59)	0.58 (0.34–1.3)	0.18	0.98
$CD4^+$ $CD45RO^-$ CXCR3/CCR4 ratio median (range)	0.93 (0.39–29.26)	3.03 (0.31–19.35)	1.83 (0.36–40.2)	0.64	0.47
Treg $CD45RO^+$ CXCR3/CCR4 ratio median (range)	0.32 (0.05–0.62)	0.31 (0.07–0.53)	0.32 (0.12–0.41)	0.53	0.60
Treg $CD45RO^-$ CXCR3/CCR4 ratio median (range)	1.27 (0.32–7.40)	1.15 (0.23–8.67)	0.92 (0.22–9.2)	0.46	0.57
$CD45RO^-$ Treg/CD4+$CCR6^+$ ratio median (range)	105.70 (42.48–311.3)	159.70 (55.19–382.9)	105.7 (42.5–311.3)	0.09	0.44
$CD45RO^+$ Treg/CD4+$CCR6^+$ ratio median (range)	2.78 (1.54–8.13)	3.46 (2.17–12.48)	2.78 (1.54–8.13)	0.09	0.60

P values indicating significant differences are shown in **bold**

3.3 Monocyte, natural killer, myeloid derived suppressor and dendritic cell subsets

Table 32. Monocyte, natural killer, myeloid derived suppressor and dendritic cell subset percentages in CF carriers compared to control and CF groups

	Control	CF	Carrier	Carrier vs Control *P* value	Carrier vs CF *P* value
N	15	17	10		
Age median (range)	28 (22–50)	25 (19–54)	34 (22–47)	0.27	0.14
Sex male (%)	5 (33%)	8 (47%)	3 (30%)		
MDSC (% non T and B cells) median (range)	0.44 (0.15–1.72)	0.36 (0.03–1.33)	0.54 (0.07–2.50)	0.72	0.51
$CD14^+$ MDSC (% non T and B cells) median (range)	0.035 (0.006–0.346)	0.047 (0.001–0.389)	0.05 (0.01–0.25)	0.50	0.98
$CD14^+$ MDSC (% MDSC) median (range)	8.27 (1.06–25.20)	15.10 (5.56–34.60)	9.68 (4.21–36.30)	0.31	0.19
$CD14^-$ MDSC (% non T and B cells) median (range)	0.40 (0.14–1.52)	0.23 (0.02–0.99)	0.38 (0.04–2.25)	0.71	0.41
$CD14^-$ MDSC (MDSCs) median (range)	91.00 (74.80–98.80)	83.30 (65.40–92.60)	90.10 (63.70–95.80)	0.30	0.17
Dendritic Cells (% non T and B cells) median (range)	12.50 (8.66–22.30)	18.90 (6.07–49.80)	14.60 (11.20–31.80)	0.07	0.31
Monocytes (% non T and B cells) median (range)	34.90 (20.00–56.10)	46.00 (24.60–76.70)	45.80 (29.2–62.7)	**0.04**	0.63
$CD16^{hi}CD14^{lo}$ Monocytes (% non T and B cells) median (range)	1.68 (0.49–4.80)	4.50 (0.65–8.37)	2.68 (0.32–4.92)	0.34	0.06
$CD16^{hi}CD14^{lo}$ Monocytes (% monocytes) median (range)	6.05 (1.24–15.70)	7.65 (1.93–22.90)	6.23 (0.59–13.50)	0.77	0.21
$CD16^{int}CD14^{int}$ Monocytes (% non T and B cells) median (range)	1.27 (0.51–2.68)	3.04 (1.47–7.04)	1.22 (0.71–3.40)	0.95	0.44
$CD16^{int}CD14^{int}$ Monocytes (% monocytes) median (range)	4.40 (1.53–6.25)	1.55 (0.59–4.24)	3.20 (1.67–5.42)	0.29	0.60
$CD16^{lo/neg}CD14^{hi}$ Monocytes (% non T and B cells) median (range)	88.80 (78.40–97.20)	37.20 (20.40–65.00)	40.90 (25.30–54.30)	0.06	0.72
$CD16^{lo/neg}CD14^{hi}$ Monocytes (% monocytes) median (range)	63.10 (40.20–76.90)	88.70 (70.20–92.70)	91.70 (81.50–96.20)	0.99	0.11
Natural Killer Cells (% non T and B cells) median (range)	49.50 (26.50–66.50)	23.10 (9.31–41.40)	35.70 (13.10–47.00)	**0.01**	0.09
$CD16^{hi}CD56^{int}$ NK (% non T and B cells) median (range)	42.60 (21.70–60.80)	18.20 (1.43–33.70)	31.30 (7.86–37.30)	**0.008**	**0.046**
$CD16^{hi}CD56^{int}$ NK (natural killer cells) median (range)	89.10 (80.20–94.90)	76.70 (15.30–91.00)	81.80 (50.60–93.60)	**0.049**	0.29
$CD16^{lo/neg}CD56^{hi}$ NK (% non T and B cells) median (range)	3.03 (1.41–3.95)	3.29 (0.68–9.27)	2.91 (1.11–8.01)	0.56	0.96
$CD16^{lo/neg}CD56^{hi}$ NK (natural killer cells) median (range)	5.94 (2.35–11.30)	16.70 (3.01–50.20)	9.33 (2.79–35.3)	**0.008**	0.17
$CD16^{lo/neg}CD56^{int}$ NK (% non T and B cells) median (range)	2.07 (0.99–7.44)	1.84 (0.38–4.48)	2.59 (1.30–3.78)	0.72	0.27
$CD16^{lo/neg}CD56^{int}$ NK (natural killer cells) median (range)	5.17 (1.90–15.00)	6.64 (3.59–48.10)	6.02 (3.32–24.1)	0.20	0.75

P values indicating significant differences are shown in **bold**

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